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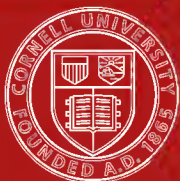
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APPLIED BACTERIOLOGY

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APPLIED BACTERIOLOGY

**STUDIES AND REVIEWS OF SOME
PRESENT-DAY PROBLEMS**

**FOR THE LABORATORY WORKER, THE
CLINICIAN, AND THE ADMINISTRATOR**

EDITED BY

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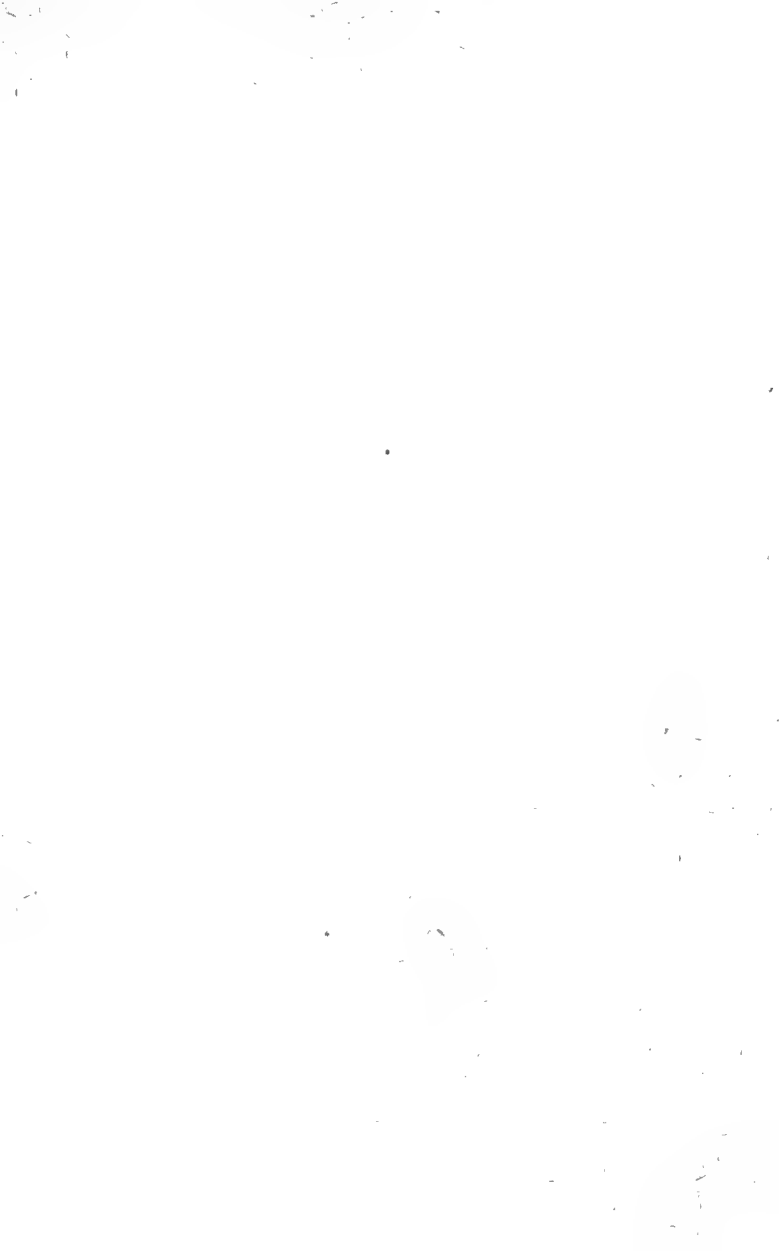
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ACKNOWLEDGMENTS

A PORTION of the matter included in this volume has already appeared in the form of separate papers ; but in almost every instance the original publications have been considerably extended here and references to important recent work on the subjects under consideration have been added. At the commencement of each section the papers which have been incorporated are mentioned. I have also been fortunate in obtaining valuable unpublished observations from Drs. J. Shaw Dunn, J. Walter McLéod, and J. W. McNee. Grateful acknowledgment is made to the *Council of the Royal Society*, also to the *British Journal of Surgery*, *British Medical Journal*, *Journal of Hygiene*, and *Journal of Pathology and Bacteriology* for permission to use work which has appeared in their pages. Toward the investigations on *Antiseptics*, assistance has been received successively from the *Carnegie Trust*, *Royal Society's Scientific Grants Committee* and *Medical Research Committee*, for which it is a pleasure to record indebtedness. The work has been carried out partly in the Pathological Department of the University

and Western Infirmary, Glasgow, and partly in the Bland-Sutton Institute of Pathology of the Middlesex Hospital. In conclusion, I cannot adequately express my sense of obligation to Professor Muir; as Head of the Glasgow School of Pathology, his teaching and encouragement have been a constant inspiration which has won the gratitude of many others beside myself. My warmest thanks are also due to the Management of the Middlesex Hospital and to the Advisory Committee of the Bland-Sutton Institute for the generous facilities which they have afforded and for their sympathetic interest in the pursuit of the work.

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14 *January*, 1918.

APPLIED BACTERIOLOGY

I

INTRODUCTORY: THE SCOPE OF APPLIED BACTERIOLOGY

By C. H. BROWNING

THESE collected studies and reviews of essentially practical subjects are intended to appeal both to laboratory workers and also to those concerned with the clinical and administrative aspects of medicine. Since clinicians and administrators will to an ever-increasing degree require the co-operation of laboratory methods for the advancement of their work, it is upon their due appreciation of the significance attaching to results furnished by laboratory investigations that the rate of progress will very materially depend.

At the present time the value of the health of the community as a great national asset has become a more prominent matter for public attention than perhaps ever before. The preservation and improvement of this possession are largely bound up with effective measures against the damage wrought

by infections. Whatever doubt may be entertained as to diagnosis being a mere academic accomplishment in the case of chronic disease in its last stages, it is certain that accuracy and rapidity in the recognition of such infective conditions as tend to assume epidemic characters is an affair of paramount importance, for it is on correct knowledge of the causes that effective measures of prevention can alone be based. In the interest of the individual patient, also, the determination of the true nature of his infection affords essential evidence on which to base specific therapy whether by antisera or by drugs or other agencies ; the field of effective operation of such remedies is likely to be greatly enlarged whenever it becomes widely accepted that systematic investigation in this domain promises much alleviation of individual disease and when the general ignorance is replaced by sympathetic interest and appreciation. This result, it may confidently be anticipated, will follow after opportunity has been taken to diffuse a knowledge of the essential problems and of the relatively great success which has already been achieved by work on such a comparatively modest scale as has hitherto been possible. Advances in therapy are, however, attended by much more expense, in every sense, than those which aim solely at improvement in diagnosis, and the latter is also the necessary precursor of rational treatment. These reasons no doubt account for the fact that investigations in pathology and bacteriology have till now been concerned mainly with the

means of recognising the causes and effects of disease and that the scientific investigation of methods of cure has been comparatively limited. Much has yet to be begun in both spheres, but it is impossible to escape the conclusion that the most rapid advances will be effected by the sympathetic collaboration of clinical and laboratory workers. The clinical observer has the great practical problems always before him, but often under circumstances, such as the advanced stage of the malady, which preclude an effective solution in the individual case ; it is one important function of the laboratory worker to discuss the complexes of disease and to attack experimentally individual aspects, bearing in mind that the ultimate aim is the prevention or modification of naturally occurring morbid processes, so that deviation from the normal will either be removed or compensated for. This result perhaps may be only remotely realisable, but nevertheless it is always the real end in view. Clearly such a scheme provides no place for " ancillary " relationships between science and practice ; it is only by mutual understanding and co-operation that the greatest success will be achieved.

In the present volume these remarks have a direct bearing on the investigations into the properties of antiseptics and also on problems of diagnosis in intestinal infections, or " enterica " as they are now often comprehensively termed. The review of the latter subject is not a colourless compilation of published work, but represents, rather, a reasoned

opinion based on conclusions reached by trained and critical independent observers. Differential diagnosis within the "enterica" group is of the highest importance, but this can only be accomplished by attention to the results already attained through the application of bacteriological and serological methods, and by an understanding of the conditions which ought to be observed in order that such investigations should yield the most fruitful returns. It must be recognised that the Widal reaction should not be the first piece of laboratory work which is demanded when there is the suspicion of typhoid or paratyphoid infection and that the examination of blood, faeces, and urine ought not to appear in the light of a reluctant afterthought. As a matter of fact, this order represents a complete inversion of the logical sequence. Investigations have shown that the occurrence of the specific organisms in the blood is one of the earliest accompaniments of the fever, and by making a blood culture at the beginning precise information as to the nature of the case can, as a rule, be rapidly obtained. To take the widest advantage of this fact, however, it is necessary that the observer of the case shall concede in anticipation that the condition may be grave and not transient; also, any lingering prejudice against the removal of a small amount of blood must be overcome. Due attention paid to the relative values of isolation of the organisms and of serological findings will in future save much of the expenditure of time and ingenuity which has been

imposed on those who require to devote attention to interpreting the evidence, in so considerable a number of instances unclear, which is obtained from agglutination tests in cases of this type, especially in individuals who have undergone prophylactic inoculation.

Particular advantage is likely to follow the conjunction of investigators when the value of the results obtained from the clinical and the laboratory sides is reciprocally appreciated; neither of the exponents should, while aware of the gaps in knowledge in his own department, demand from the other one hundred per cent. of unequivocal observations! At the present time any material increase in the accuracy of diagnosis must be welcomed.

In connection with the identification of organisms belonging to the typhoid-coli class a brief, but highly significant series of observations on immunity reactions in the coli group has been added. Immunity reactions such as agglutination, precipitation, and complement fixation have been found so highly specific and so uniform in their occurrence in certain standard instances, as for example in the recognition of typhoid bacilli and the identification of the species-origin of serum proteins, that the danger has arisen of generalising and thus hastily assuming that information capable of simple interpretation regarding the relationships of other allied organisms may be yielded by similar methods. The work shows that this is far from being necessarily the case and affords a useful warning that prema-

ture generalisations may involve in discredit these valuable biochemical reactions. In order to bring into even greater prominence the dangers of indiscriminate generalisation in this field, a general review of the chemistry of antibody reactions has been added, for there is almost no line of biological inquiry in which a more judicial attitude is necessary if fallacy is to be avoided.

The creation of an immense army and the importance of preserving its health has led to the multiplication of routine laboratory work in a degree never anticipated and the success attained has been nothing short of phenomenal. Methods which will secure the desired end with the greatest precision and at the least cost of time and materials are a desideratum in all forms of repetitional or routine work, whether for commercial purposes of production or for diagnosis in epidemic disease, and the need for such methods has never been so well recognised as now. Thus the present volume includes a detailed account of the use of differential anti-septics for the solution of that most difficult bacteriological problem, the isolation of small numbers of pathogenic organisms from mixtures of similar bacteria, especially the recovery of typhoid-paratyphoid bacilli from faeces. The procedure involving the use of brilliant green and telluric acid was, in its present form, first devised as the result of work carried out in Professor Muir's Department at the University and Western Infirmary, Glasgow, and subsequently continued at the Bland-Sutton Insti-

tute of the Middlesex Hospital. The great measure of success which has attended its use under the exigencies of active service has encouraged this attempt to give it wider publicity. Other applications of selective antiseptic action dealt with include the isolation of *B. diphtheriae* by means of telluric acid.

It has not been considered out of place to state comparatively fully the experimental work on which these practical methods are based. Even though the resulting procedure becomes one of mere routine, we hold strongly that it should be at all stages controlled, if not actually practised, by a scientific worker skilled in the subject and not by a mere technician ; therefore we feel that methods will be successfully applied only in so far as their rationale is appreciated. No doubt efficiency in the commercial sense is usually secured by means of highly complex automatic machinery and the human element is reduced to some essential, but relatively simple series of movements, the products of such work being then tested, again often by automatic devices which make little or no demand on the judgment of the operator. In bacteriological procedures, however, there is little room for machinery ; the manipulations are relatively simple, but they call for a reasoning attention to detail. Where also individual human welfare depends on the results, as in the diagnosis of disease, statistics are not the sole aim and the proportion of failures or mistakes cannot be regarded with equanimity as inevitable ;

the possible significance of any error to the particular patient concerned must always remain before the mind, and if the mistake were avoidable the burden of responsibility for evil consequences would indeed be a heavy one. Only a responsible judgment is likely to produce the best results under these circumstances. Further, there are few branches of the subject, if any, in which even an approach to finality can be said to have been reached. Hence we believe that a permanent standard of excellence in such investigations can be attained only by employing highly trained and widely experienced workers who have a large part of their time free for original research, to which they can apply themselves equipped with the perspective gained from a varied routine and a perception of the problems still unsolved. Mere rapidity of output may not reach its utmost development in this fashion, but there can be no doubt that progress and, in its truest sense, efficiency lie along these lines.

II

THE DIAGNOSIS OF "ENTERICA" INFECTIONS BY BACTERIOLOGICAL AND SEROLOGICAL METHODS

BY C. H. BROWNING, T. J. MACKIE, AND
L. H. D. THORNTON

THE importance of diagnosis in cases of intestinal infection is threefold, (*a*) as a guide to treatment especially where specific therapy is available, *e.g.* in amoebic and bacillary dysentery, (*b*) to enable prophylactic measures to be taken against epidemic spread, and (*c*) as a means of determining the efficiency of prophylactic inoculation. No doubt when the symptomatology of these diseases is typical a trained clinical observer may be able to make a diagnosis with great certainty; but it is necessary to recognise the limitations of the clinical method in this connection.¹ (See Bertrand Dawson and

¹ *Marris's phenomenon* occupies a special place, as the evidence indicates that it depends on a specific effect of the toxins of the typhoid and paratyphoid organisms, since the test is also given shortly after typhoid or paratyphoid inoculation; it consists in the fact that acceleration of the heart fails to occur after a subcutaneous injection of atropine when there is an early infection with typhoid or paratyphoid organisms (see p. 49).

others.) Bacteriological and serological studies have proved that the mild and atypical cases frequently form a large proportion, and perhaps the great majority, of the total ; it is impossible to determine these on a purely clinical basis, yet they are extremely important because they may disseminate the infection at an early stage and, also, later a considerable proportion of chronic carriers is recruited from this group of mild infections. Further, although typhoid and paratyphoid infections may be capable of differentiation statistically by clinical features, yet this permits only of a presumptive diagnosis in the individual instance and the same is even more true of the dysenteries. Accordingly, bacteriological examination should constitute a routine procedure in the investigation of every febrile case whether or not intestinal symptoms are prominent. It is a point which cannot be too clearly emphasised that *where there is " fever " blood cultures should be made at the earliest possible moment ;* if this were done as a routine when the case was first seen much subsequent uncertainty in diagnosis would be obviated and in all probability only an insignificant minority of cases of typhoid and paratyphoid infection would remain undiagnosed. That blood culture can be carried out practically on a very extensive scale is shown by Leboeuf and Braun, at a French military hospital ; in the course of a year they carried out 12,028 such cultures and during the latter half of that period the blood of every febrile case entering the hospital was so

examined. The plan of waiting for the disease to declare itself with the lapse of time must be recognised as highly injudicious both from the point of view of the welfare of the individual patient and also of the general community. The bacteriological examination of the faeces and urine should be performed repeatedly, and, according to some observers, the specific organism can frequently be obtained by cultures from the fauces.

The isolation of the specific organism constitutes the only conclusive evidence of infection. Of course, it does not exclude the presence of other infections and it does not prove that the organism is at the time necessarily causing either a part or the whole of the pathogenic effects. When a specific organism known to be usually pathogenic is isolated from a given patient, the presumption is, however, very strong that it is, in part at least, responsible for the symptoms and, of course, the infected individual is a possible centre from which the disease may be disseminated.

The Widal reaction with the patient's serum affords valuable information which is, however, subject to several serious restrictions. (1) A natural infection with *B. typhosus*, etc., is commonly much inferior to artificial immunisation with dead cultures as a means of producing agglutinins ; hence the absence of the phenomenon or a weak reaction does not effectively exclude infection. On the other hand, a powerful reaction is of great value, especially when it is found to develop in the course of the illness,

This applies where the individual has not previously been the subject of infection with the organism in question and also has not been vaccinated with it in the form of dead cultures, etc. (2) In the case of patients previously infected or vaccinated, especially if these events are of recent date—within a year or thereby—a subsequent infection with a different organism is capable of causing variations in the specific agglutinins developed by the original infection or injection, which probably cannot be accurately foretold, since peculiarities of the individual have in this respect an important modifying effect. Hence variations in the agglutinating power of the serum of a person previously vaccinated or infected with the organism in question, while possibly of statistical value in determining the nature of an illness which has developed subsequently, are liable to be fallacious in the individual case, even when considerable in degree. The deduction which may be drawn from a large amount of work on the problem is that the biological data of agglutination in the human subject are often obtained late and are difficult to interpret, and that even a practical approach to universally satisfactory results cannot be secured. It is more scientific as well as more useful practically to recognise that a considerable proportion of cases must remain serologically doubtful and to concentrate efforts on means to ensure the isolation of the infecting organisms, a point which has also been emphasised by Ledingham and Penfold. This is especially true in the case of inocu-

lated individuals. It may appear that the defects of the Widal reaction as a diagnostic method have been somewhat laboured here; but it is necessary to remember that in the past this test has occupied a disproportionately large place in general estimation, and has frequently been relied on as the sole laboratory criterion of typhoid infection.

ISOLATION OF TYPHOID-PARATYPHOID BACILLI BY BLOOD CULTURE

This is most likely to prove successful at the commencement of fever, during the first week with *B. typhosus*, but perhaps also later, and especially in the earlier part of the first week with the paratyphoid bacilli; also in relapses with all three organisms, especially at the commencement (see p. 43).

Method

Sterilise the skin by any of the ordinary methods; McLeod (unpublished) has proved that a solution of 0·5 per cent. each of brilliant green and methyl violet in 50 per cent. spirit is specially suitable for this purpose; 10 c.cm. of blood are withdrawn from a vein by means of a syringe¹ and 5 c.cm. each are used to inoculate (a) 50 c.cm. peptone water or broth and (b) 10 c.cm. sterilised ox bile or 50 c.cm. of $\frac{1}{2}$ per cent. bile salt plus 1 per cent. sodium citrate in water (Cummins' medium). The latter has been found to yield cultures much more rapidly than bile. The mixtures should be incubated for a week before finally concluding that no growth will occur. Daily a hanging drop of the culture, or simply a drop between slide and cover-glass, should be examined and if organisms are seen a sub-culture should be made on MacConkey's lactose-bile-salt-neutral-

¹ Syringes may be sterilised by boiling in water; hot oil, as commonly employed, is not effective.

red agar ¹ and also one on ordinary agar, which is utilised for the differential examination. Finally a subculture should be made whether organisms are seen in the hanging drop or not. If contamination with streptococcus (enterococcus) has occurred, as is frequent under military conditions, a preliminary subculture into peptone water containing brilliant green (1 : 200,000) prior to plating on MacConkey's medium readily effects separation (McNee).

Organisms other than members of the typhoid-paratyphoid type which may be isolated from the blood in enterica.—Systematic blood culture leads to the recovery of a wide variety of organisms, e.g. *B. coli*, *B. faecalis alkaligenes*, a non-motile Gram-negative cocco-bacillus without fermentative properties (but which later on, in artificial cultures, may ferment glucose without gas-formation), *B. Morgan* No. 1, a bacillus differing culturally from the last-named in that it forms no indol ("C.L.A.1"), paracolon bacilli, also proteus and pyocyaneus types. These organisms cannot of course be regarded as primarily responsible for the disease; they probably

¹ The bile salt supplied commercially varies in its inhibitory power on bacterial growth and it may be advisable to employ 0·25 per cent. in place of 0·5 per cent. originally recommended. The following simple method shows whether the medium is unduly inhibitory: Make a slightly opalescent suspension of *B. typhosus* in saline and from this prepare dilutions of 1 : 10, 1 : 100, 1 : 1000 etc.; then make a stroke of each dilution on a plate of ordinary agar and of bile-salt medium respectively; incubate for twenty-four hours at 37° C., and note the abundance of growth on the two media. It will be found that the inoculation with a particular dilution of typhoid suspension gives on agar closely adjacent, but not confluent, colonies; the same dilution should yield a similar abundance of growth on a satisfactory bile-salt medium.

represent a secondary blood infection which is predisposed to wherever ulcerative lesions of the intestinal tract exist, as in the various forms of dysentery. It is a fact that in such ulcerative conditions organisms of the above-named types tend often to occur in large numbers in the bowel.

FROM FAECES AND URINE

As regards the *faeces*, the occurrence of typhoid bacilli has been found to be most frequent in the first three weeks of the illness, especially the third week. In paratyphoid infections the maximum occurs earlier—in paratyphoid A in the first and second weeks (see Krumwiede), especially about the twelfth day, in paratyphoid B. cases at the end of the second and beginning of the third weeks. Thus the bacilli appear to be most readily found at about the commencement of defervescence. Of course, while these are the periods to be chosen if only a single examination is made, repeated tests should if possible be carried out at short intervals—not over a week—in all cases until the bacteriological diagnosis is established. For the *method of isolation*, see p. 113.

Urine.—Bacteriological examination is a valuable diagnostic procedure when *repeatedly* carried out. There appear to be transient bacilluric periods in enterica infections ; hence daily examination (plating out of two or three loopfuls on a MacConkey plate) over a period of seven to ten days will lead to the isolation of the infecting organism in a large proportion of cases. Thus in a series of twelve test

cases examined during the second and third weeks of illness, the specific organism was recovered in each (*Methods of Isolation*, see p. 133).

When isolated pale colonies have been obtained on a differential solid medium, such as MacConkey's, subcultures are made on agar so as to obtain pure growths for subsequent tests.

As a preliminary to subculture a portion of the colony may be rubbed up on a slide in a drop of a dilution of a powerfully agglutinating antiserum in 0.5 per cent. carbol saline; in a few minutes the drop is examined with the low power, the condenser being well stopped down. Two agglutinating mixtures may be used—(1) of equal parts of potent antisera to *B. typhosus* (diluted 1 in 50), *B. paratyphosus* A (1 : 50) and B (1 : 50) and (2) of *B. Shiga* (1 : 100) and *B. dysentery* Y (1 : 100). Agglutination is significant, but no organism should be excluded from further examination owing to a negative result in this rough test.

In the case of direct plate cultures of faeces, a number of pale colonies should be tested (three to five at least); no great reliance should be placed on the naked-eye appearance of colonies, as this is a most variable character. In the case of *B. paratyphosus B* colonies, however, a striking feature consists in the formation of a raised opaque border, which develops when the plate culture, after incubation for eighteen to twenty-four hours at 37° C., is allowed to remain for a further period of twenty-four hours at room-temperature. This character, while not absolutely specific for *B. paratyphosus B*, is so highly distinctive as often to enable scanty colonies of this organism to be picked out with certainty from a plate culture so rich in other non-

CULTURE REACTIONS OF THE TYPHOID-PARATYPHOID-DYSENTERY GROUP AND OF CERTAIN ORGANISMS RESEMBLING THESE

Bacillus.	Mo- tility.	Glucose.	Lactose.	Saccharose.	Mannite.	Dulcitol.	Maltose.	Indol.	Gelatine.	Litmus Milk.	Remarks.
Typhosus	+	↓	—	—	↓	— (may be ↓ later)	↓	—	—	a	} May be non-motile when recently isolated; also inagglutinable, but later agglutinated practically to the limit by antisera to genuine strains.
Paratyphosus A	+	+	—	—	+	+	+	—	—	a (alk. very late)	
Paratyphosus B	+	+	—	—	+	+	+	—	—	a (later alk.)	
Enteritidis Gaertneri	+	+	—	—	+	+	+	—	—	a (later alk.)	Differs from Para. B only in agglutination.
Suipestifer (B. Aertryck)	+	+	—	—	+	+	+	—	—	a (later alk.)	Differentiated from Para. B by agglutinin absorption.
Morgan No. 1	+	↓	—	—	—	—	—	+	—	alk. after 15 days	
Faecalis alkaligenes	±	—	—	—	—	—	—	—	—	alk. 1st day	
Coli anaerogenes	+	↓	↓	↓	↓	↓		+	—	a	
Coli mutabilis	—	+	(later +)	—	+	—		—	—	a (later a c)	
Paracoli types	{ ± ±	{ + +	{ — —	{ — —	{ + +	{ — +	{ + +	{ ± +	{ — —	{ a (later alk.)	
Dysenteriae Shiga	—	↓	—	—	—	—	—	—	—	a (later alk.)	Agglutinated by antiserum to genuine strain.
„ Flexner	—	↓	—	—	↓	—	↓	+	—	a (later alk.)	} Agglutinated practically to the limit by antiserum to genuine Y-strain.
„ Y	—	↓	—	—	↓	—	—	±	—	a (later alk.)	
„ Strong	—	↓	—	↓	↓	↓	↓	+	—	a (later c)	Not agglutinated by antiserum to Y-type.
„ Thomson and Mackie (2)	—	↓	—	—	—	—	—	+	—		} Not agglutinated by anti-Shiga or anti-Y serum.
„ „ „ (4)	—	↓	—	—	—	—	↓	+	—		
„ „ „ (30)	—	↓	—	(later ↓)	↓	(later ↓)	—	+	—		
„ „ „ (40)	—	↓	(later ↓)	—	↓	—	↓	+	—		

+ = acid and gas } in fermentation tests.
 ↓ = acid, no gas
 — = no change

+ in motility column = motile.
 — „ „ „ = non-motile.
 ± = variable behaviour.

a in litmus milk = acid.
 c „ „ „ = clot.
 alk. „ „ „ = alkaline.
 — in gelatine column = no liquefaction.
 + in indol column = indol formed in peptone water.
 — „ „ „ = no indol formed in peptone water.

Note (1).—B. typhosus forms acid from sorbite and so differs from B. dysenteriae Shiga, which does not (of value in case of a non-motile typhosus strain).
 Note (2).—B. paratyphosus-enteritidis group all—except Para. A—ferment xylose; but presumably pathogenic non-xylose-fermenting strains have been found, which are not agglutinated by anti-A serum; all ferment rhamnose and so differ from B. typhosus, which does not. (See Krumwiede, Pratt, and Kohn, Journ. Med. Res., vols. xxxiv, 1916, p. 355, xxxvi, 1917, p. 509.)
 True paratyphoid strains are said not to ferment salicin and so differ from organisms otherwise resembling paratyphoid A and B bacilli, but which are not agglutinated by antisera to genuine strains and which generate antisera devoid of agglutinating action on the latter.

lactose fermenters that otherwise they would in all probability have been missed. In the case of cultures made from brilliant-green medium all the pale colonies from a given tube are likely to be of the same organism unless in occasional cases of multiple infection.

IDENTIFICATION OF BACILLI

When a pure culture of Gram-negative bacilli has been obtained, the organism should be examined for its biological reactions as specified in the table opposite; several types of coliform bacilli are included here, the reactions of which might suggest that they were members of the typhoid-paratyphoid series if an incomplete examination were made.

Certain practical points in connection with these tests are important and will now be treated more in detail.

FERMENTATION REACTIONS, ETC.

Fluid medium with Durham's tubes must be used (solid medium is valueless). The medium is prepared as follows :

(A). 1 per cent. peptone¹ and 0·5 per cent. NaCl in water are autoclaved and filtered, then 0·25 per cent. of a 1 per cent. watery solution of neutral red is added and the reaction adjusted by the addition of a dilute watery solution of NaOH (about 1 per cent.) till the tint is just not red, *i.e.* is neutral, as tested by observing the colour of the solution in a test-tube. Sterilise in bulk, preferably in the autoclave. (B). Sterilise the carbohydrate, preferably in the autoclave at 105° C. for ten minutes, in the

¹ Savory & Moore's peptone has been found satisfactory.

form of a 10 per cent. watery solution in sufficient amount to make a 1 per cent. solution when added to (A). Then mix A and B and distribute in test-tubes containing Durham's tubes, all previously plugged and sterilised. Finally resterilise in the autoclave for ten minutes at 105° C. or twice in the Koch steriliser for twenty minutes. Where economy in time and materials must be specially considered, small test-tubes, measuring $4 \times \frac{1}{2}$ in., may be used and the inverted gas-tubes are made of small pieces of narrow quill tubing sealed at one end. In order to exclude error from the use of impure sugars, a useful practical test consists in inoculating two tubes of medium containing each sugar, (1) with an organism which ferments it, and (2) with one which does not, but which ferments the most likely contamination: *e.g.* to test lactose medium, inoculate it with *B. coli* and *B. typhosus*; test saccharose with *B. MacConkey* No. 71 and *B. coli communis* (Escherich).

These cultures should be incubated for a week before making the final reading.

It is noteworthy that the fermentation reactions of a culture of the typhoid-coli group should not be taken as definitely determined until the organism has been grown on artificial culture medium for some time and has been frequently subcultured. The capacity to ferment certain substances, *e.g.* lactose, is not manifested by some organisms until they have become habituated to artificial culture media, and both time and repeated subculture are factors. Diagnoses based on the behaviour of newly isolated organisms may be responsible for troublesome errors in this respect; very frequently, however, three or four subcultures extending over a period of a week altogether will enable the stable characters of the organism to develop.

It is of practical importance to differentiate (1)

weak fermentative characters, which may be manifested by recently isolated organisms, and which are comparatively soon replaced by more stable characteristics, *e.g.* the acquisition of lactose-fermenting power by a strain after several subcultures on agar, which in the early generations did not alter lactose; absent or deficient gas-formation from dulcitate in the case of recently isolated *B. paratyphosus* A is also an important practical example; (2) mutations involving the acquisition of power to act on a given sugar after more or less prolonged growth on media containing the particular carbohydrate; the latter is not likely to prove a disturbing factor in practical work, except in the case of *B. typhosus* which, as Penfold pointed out, may form acid in dulcitate media after growth for some days. Attention to these points will obviate such fallacious conclusions as that (1) paratyphoid bacilli form indol (see Jordan), or (2) an organism otherwise of the paratyphoid A type is to be excluded because it has not fermented dulcitate with gas-formation.

Unusual Organisms.—In Faeces: It is to be noted that a variety of unusual types of “Coliform” or “Paracolon” bacilli occur in the faeces in intestinal disorders and in the urine in typhoid infections. Thus in the *faeces* of enterica cases, on occasion, the following organisms have been found in large numbers, *B. Morgan* No. 1 and an organism with similar cultural characters, but which forms no indol (“C.L.A.1”), *B. faecalis alkaligenes*, paracolon bacilli, pyocyanus types, also sometimes streptococci

in enormous numbers ; it has already been noted (see p. 14) that the majority of these may also invade the blood stream. Certain of the coli types may often be recovered with special ease from the faeces by the brilliant-green method, as Mackie found (see p. 211), and an elaborate study has since been made by Douglas and Colebrook of certain of these organisms in convalescent dysenteric cases from the East. *In Urine*: Patrick's observation that such organisms occasionally appear in pure culture in the urine of cases of typhoid fever in the later stages, certainly points to their possessing pathogenic characters, but there is not yet any definite basis for considering them to be specifically concerned in the causation of epidemic intestinal affections. In enterica cases a variety of organisms may be responsible for bacteriuria, in addition to the specific types and to those paracolon bacilli just mentioned, viz. the bacillus referred to as "C.L.A.1," an organism differing from B. Morgan No. 1 in being non-motile ("C.L.A.3"), a non-motile cocco-bacillus (also found in blood and faeces), and streptococci.

Motility should be examined for by making a hanging-drop or a simple wet preparation between slide and cover-glass from peptone-water cultures or the condensation water of agar cultures, which have been incubated at 37° C. for six hours ; when the culture becomes older motility may diminish greatly. Dark-ground illumination is useful in examining for motility. In order to determine that

an organism is motile definite movements of translation across the microscope field in various directions must occur ; this must be distinguished from (1) streaming movement where all the organisms flow in one direction, (2) brownian movement, *i.e.* oscillation back and forth and around a given point, but with no definite translation. Occasionally a strain of *B. typhosus* may show no motility for a long period after isolation.

When an organism conforms to one of the standard pathogenic types in culture reactions its behaviour in agglutination tests should be examined with powerful artificial antisera derived from animals. (The use of human sera from cases of natural infection is absolutely valueless for this purpose.) In this connection it is important to note that while an organism may be at first deficient in fermenting power or motility and should not be immediately excluded on that account, on the other hand, any *excess* of characters at once disqualifies it as a member of the genuine typhoid-paratyphoid group.

Some workers prefer to abbreviate the series of fermentation tests and for purposes of economy in time or materials pass at once to agglutination reactions. It is very doubtful whether in the long run this is a wise plan, since recently isolated strains are liable to be abnormal in some character, but the following procedure may be adopted after pale colonies on a MacConkey plate have been obtained :

Abbreviated Scheme of Identification

- I. Inoculate from a pale colony one tube each of

(a) lactose neutral red peptone water	}	and place at 37° C.
(b) mannite neutral red peptone water		
(c) sloped agar, including its condensation water		
- II. Examine condensation water of agar culture after six hours for motility, return culture to incubator.
- III. Next day observe (a) and (b)—
 - If (a) shows acid or acid and gas, reject the strain.
 - If (a) shows no change and (b) shows acid only, test agglutination with anti-typhoid serum if test II showed motility or with anti-dysentery serum if non-motile; but *note* (1) the organism may be a late lactose-fermenter and
 - (2) *B. typhosus* is occasionally non-motile.
 - If (a) is unaffected and (b) shows acid and gas, test agglutination with anti-paratyphoid A and B and also anti-Gaertner sera.

AGGLUTINATION

In general, it is essential for the identification of *B. typhosus* and paratyphosus A and B that they should be agglutinated in high dilutions by potent antisera derived from animals, usually rabbits, immunised with classical strains. Immediately after isolation the cultures may be insusceptible to agglutination, so that, as a rule, no conclusion as to inagglutinability should be drawn till after the organism has been isolated for some time and has been frequently subcultured. Rarely inagglutinability persists for a long period (McIntosh and M'Queen) and may be accompanied by lack of motility. If an organism which conforms in all other characters with *B. typhosus* or paratyphosus fails to agglutinate, it should not be rejected until after the lapse

of time and repeated agglutination tests made after many subcultures. The nature of an in-agglutinable strain, *e.g.* of *B. typhosus*, is proved when on using it to immunise an animal, an immune serum is generated which is powerfully agglutinating for a typical strain of typhoid bacillus. The nature of inagglutinable strains may also be determined by agglutinin absorption tests (see p. 28). It is of great practical importance for their identification that the typhoid-paratyphoid bacilli are so stable and uniform in their serological characters; in the case of many other organisms belonging to the coli group this is not the rule and an antiserum may agglutinate only the individual strain in response to which it was developed, although, on the other hand, *a heterologous organism with widely different fermentative characters may be agglutinated more powerfully than the homologous bacillus which was used to generate the antiserum* (see Mackie, p. 204). The possibility of such *paragglutination* should never be lost sight of, otherwise completely erroneous conclusions as to the relationships of organisms may be arrived at. Again, an organism cannot be identified, say as *B. typhosus*, merely because it is agglutinated in considerable dilution by an antityphoid serum; thus Frost has recorded a motile Gram-negative bacillus obtained from water, of proteus type, which liquefied gelatine and serum and in its other characters differed markedly from *B. typhosus*. This organism, although not agglutinated by the serum of a normal

rabbit, was agglutinated almost as actively as *B. typhosus* when in response to immunisation with the latter organism 1 : 500 of the serum agglutinated *B. typhosus* ; later, however, when *B. typhosus* was agglutinated at a dilution of 1 : 20,000 the proteus strain was agglutinated only at 1 : 1000 and not in 1 : 5000.

Pseudo-paratyphoid Bacilli.—Various workers have isolated organisms from the faeces of enterica cases, which give all the cultural reactions of *B. paratyphosus* B, but which are not agglutinated by antisera to *B. paratyphosus* B, Gaertner's bacillus, or *B. Aertryck*. A bacillus of this kind was described by Savage and designated *B. pseudo-Gaertner* ; an important point in connection with these organisms is that, so far as we know, they have never been isolated from blood cultures. Hence there is no justification for adding any further members to the classical typhoid-paratyphoid A and B types. This pseudo-paratyphoid bacillus has not uncommonly been found in large numbers in direct cultures from the faeces in dysentery cases ; when present it tends to be enriched by brilliant green.

Preparation of Agglutinating Sera

Rabbits receive intravenous injections at weekly intervals of a suspension in saline of a twenty-four-hour agar culture of a standard strain killed by heat (half-hour at 65° C.). The following doses may be given, $\frac{1}{20}$, $\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{2}$, 1, and 2 cultures.¹ Ten to

¹ In the case of *B. dysenteriae* Shiga it is necessary to begin with smaller numbers of organisms, not above 20,000,000, and the highest potency of the serum does not usually exceed 1 : 1000. For this and other important points in connection with the production of agglutinating sera, see Chick, *Lancet*, i. 1917, p. 857.

twelve days after the last injection a specimen of blood is withdrawn from the ear vein, and the serum, after separation and heating for one hour at 56° C., is tested. If it is powerfully agglutinating (marked sedimentation of the hemelogenous organism in twenty-four hours produced by high dilutions of the serum, e.g. 1:4000 or higher) the animal is bled and its serum is stored in amounts of about 1 c.cm. in quill tubes drawn out into capillary points at either end (see p. 55); after filling, the ends are sealed in the Bunsen flame and the serum is heated at 56° C. for one hour on two or three occasions. In preparing the serum due aseptic precautions should be taken at all stages, since the agglutinating power is apt to decrease if it is frequently heated at 56° C. Its potency should be controlled from time to time, especially in a warm climate; in a temperate climate such agglutinating serum may alter little in the course of several years.

Method of carrying out the Tests

A well-grown twenty-four-hour agar slope culture of the organism to be tested is emulsified in about 3 to 5 c.cm. saline and allowed to stand for half an hour at 37° C. (density of 1000–2000 million per c.cm.). The supernatant emulsion is then decanted off; thus grosser particles are removed. A convenient standard of opacity for the bacterial emulsions is 0·1 per cent. suspension of very finely powdered pure chalk in distilled water (Mackie and Wiltshire). For safety in working, saline containing 1:1000 formalin may be employed. It is important that the bacterial emulsion should not be too thin; this point can be readily verified by setting up a series of agglutination tests in which the series differ only in the fact that increasing dilutions of the bacterial suspension are employed; it will be seen that a thin suspension will show no sedimentation with a concentration of serum which causes very marked sedimentation of a dense suspension. Garrow takes advantage of this fact by using very dense suspensions (10,000 million organisms per 1 c.cm.) in his rapid clinical agglutinator. A series of dilutions of the antiserum are prepared of double the strength of the concentration ultimately desired,¹ e.g. if it is intended to test the effect of the serum in

¹ 1 c.cm. and 0·1 c.cm. pipettes of suitable pattern are the instruments best suited for such measurements (see p. 52).

concentrations of 1:100, 1:200; 1:400; 1:800; 1:1600, 1:3200, 1:6400, 1:12,800, double these strengths are first prepared; thus, eight small test-tubes ($3 \times \frac{1}{2}$ in.) are placed in a rack¹ (see p. 56), and the constituents are added in the order indicated:

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(i) Normal saline	c.cm. 4.9	c.cm. 2.0	c.cm. 2.0	c.cm. 2.0	c.cm. 2.0	c.cm. 2.0	c.cm. 2.0	c.cm. 2.0
(ii) Anti-serum	0.1							
(iii)	mix	2.0 of (1)	2.0 of (2)	2.0 of (3)	2.0 of (4)	2.0 of (5)	2.0 of (6)	2.0 of (7)
		mix	mix	mix	mix	mix	mix	mix

A. The antiserum is measured with the 0.1 c.cm. pipette; for the other measurements the 1.0 c.cm. pipette is used. At stage (iii) begin by transferring 2 c.cm. of (1) into tube (2); then mix by drawing up into the pipette and blowing back the contents of tube (2) several times; then add 2 c.cm. of (2) to (3), mix; and so on.

B. To each of a series of nine small test-tubes ($3 \times \frac{1}{2}$ in.) in a rack add $\frac{1}{2}$ c.cm. bacterial suspension; make as many of these series as there are cultures to be tested. When examining unknown organisms it is advisable to include a known typical strain. The amounts shown above suffice for three series of tests.

C. To No. (9) add $\frac{1}{2}$ c.cm. of saline; then to (8), (7), (6)–(1) respectively add $\frac{1}{2}$ c.cm. each of serum dilutions (8), (7), (6)–(1); the result is a suspension of organisms in the following concentrations of serum:

1	2	3	4	5	6	7	8	9
1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	0

No. 9 is the control to show that the organism does not sediment in the absence of serum.

Place the rack of tubes in the incubator at 37° C. and allow to remain for two hours—the appearance of agglutination is hastened by removing the tubes from the incubator at intervals of about half an hour and allowing them to stand at room

¹ “Plasticine” is to be avoided as dirty and wasteful of time.

temperature for ten minutes. The convection currents which are set up as the fluid cools often cause agglutination to appear suddenly. (One of the most rapid methods of producing agglutination of an organism mixed with its specific antiserum, is to roll the tube—held in the vertical position—vigorously between the palms of the hands.) A preliminary reading may be taken at the end of two hours, but it is best, before making the final reading, to wait until the tubes have stood overnight at room temperature, as agglutination often progresses markedly during the latter period.

Results are conveniently recorded as follows (in making the readings hold the tubes between the observer and a bright window or lamp with a dark background at some distance):¹

- ++++ or (4) = complete sedimentation; supernatant fluid perfectly clear;
- +++ ,, (3) = practically complete sedimentation; supernatant fluid almost clear;
- ++ ,, (2) = marked sedimentation; supernatant fluid opalescent;
- + ,, (1) = only slight or negligible sediment as compared with control; the supernatant fluid is distinctly turbid, but in it fine flocculi are just visible to the naked eye;
- ,, (0) = control; no sediment and no flocculi visible in the fluid.

+ may be taken as the limiting concentration which causes agglutination just visible to the naked eye, *i.e.* “ Standard agglutination ” in Dreyer’s nomenclature (the use of a magnifying glass tends to confuse rather than to aid the reading).

It is important when working with living organisms and potent agglutinating sera to keep a water bath (a fish kettle containing soft or distilled water) constantly boiling on the work bench, so as to kill the organisms and inactivate traces of agglutinating serum in the pipettes between operations; if several pipettes are used one may be laid on a sheet of paper to cool while

¹ Where numerous agglutination tests require to be read a simple permanent apparatus can be made in the form of a box with blackened interior, with an electric lamp in the roof, the direct rays of which are shaded from the observer’s eyes.

another is in use. Some workers prefer to have a beaker of water which is kept boiling and one filled with cold saline; the pipette after use is then rinsed alternately with cold, boiling, and cold.

If the homologous organism is agglutinated *one plus* in 1 : 6400 and *minus* in 1 : 12,800, then a recently isolated unknown organism which is under examination, may be accepted as likely to be serologically identical if it shows *one plus* in the next greater concentration, *i.e.* 1 : 3200 and *minus* in 1 : 6400; if it shows *one plus* only at 1 : 1600 and *minus* at 1 : 3200, the result may possibly be due to coagglutination and cannot be accepted as "clean"; it should be investigated again after further subcultures and if its agglutination does not then approach more closely to the homologous organism the result must be regarded as doubtful. Difficulties arising from a slight degree of agglutination on the part of an unknown organism, as compared with the control, are more likely to occur in the paratyphoid group than with *B. typhosus*, since organisms giving the fermentation tests of the latter are usually true *B. typhosus* and are also identified with ease by agglutination. Passage through brilliant-green and telluric-acid medium has not been found to affect notably the agglutinating properties of these organisms.

Absorption of Agglutinins

If a serum agglutinates a given species of organism X, this may be due to a specific immunity response resulting from inoculation or infection with X; on

the other hand, it may be due to *co-agglutinins* developed in response to immunisation or infection with an allied species of organism Y. To determine which of these two possibilities represents the truth in a given instance, recourse may be had to absorption of agglutinins in the following fashion :

- I. (a) Treat the serum with a suspension of bacillus X ; then centrifuge and recover the fluid = *serum-treated X*.
 (b) Do ditto with Y = *serum-treated Y*.
- II. (a) Test the agglutinating power of serum-treated X for bacillus X and bacillus Y.
 (b) Test the agglutinating power of serum-treated Y for ditto.

The possible results and the conclusions to be derived therefrom may be represented thus :

Reagent.	Tested on Bacillus	Result.	Conclusion.
Serum-treated X .	{ X	no agglutination	{ X is the infecting
	{ Y	no agglutination	
Serum-treated Y .	{ X	agglutination	{ organ-ism.
	{ Y	no agglutination	
Serum-treated X .	{ X	no agglutination	{ Y is the infecting
	{ Y	agglutination	
Serum-treated Y .	{ X	no agglutination	{ organ-ism.
	{ Y	no agglutination	

That is to say the *homologous* organism removes *both the main agglutinins and also the co-agglutinins* ; on the other hand, the *heterologous* organism removes the *co-agglutinins only* ; this is known as *Castellani's reaction* (for a practical application see p. 39).

In practice it is important to use large amounts of bacteria for treating the serum and to dilute the agglutinating serum to a strength which is not too far removed from the limiting concentration which just suffices to cause marked agglutination ;

e.g. if a serum agglutinates markedly up to 1 : 5000, then the dilution *e.g.* of 1 : 500 may be treated with the bacterial suspension for an hour at 37° C. ; a series of dilutions of the treated and untreated serum should be tested in the usual manner.

Absorption of agglutinins may be practised for three other purposes: (1) to identify an inagglutinable (usually recently isolated) genuine strain, *e.g.* of *B. typhosus*, which will be found to absorb the agglutinins from antityphoid serum just as an agglutinable strain does; (2) agglutination of a culture might possibly occur in serum, but not in the control suspension in saline; if the agglutination is not due to the specific serum agglutinins, then the latter will not be absorbed by the organisms; (3) to differentiate strains of organisms which are very closely allied, *e.g.* *B. paratyphosus* B and *B. suipestifer*; thus Bainbridge and O'Brien found that while an antiserum to *B. paratyphosus* B agglutinated that organism and *B. suipestifer* to an equal extent in series of dilutions, each of these organisms removed all the agglutinins for both species from the homologous antiserum, whereas an antiserum if treated with the heterologous organism was deprived of its agglutinins for the latter culture alone and retained its agglutinins for the type of organism which had generated it. In employing the test for these purposes it is also important to dilute the serum sufficiently prior to absorption.

THE WIDAL REACTION WITH THE PATIENT'S SERUM

Given a case which has been ill sufficiently long to enable antibodies to be produced, *i.e.* a week or more, the presence in increased amount of agglutinins in the serum for one of the typhoid-paratyphoid bacilli affords strong presumptive evidence that there is infection with the organism in question. If in the course of the acute stage the agglutinin content of the serum for one organism undergoes marked increase as determined by repeated examinations at intervals of about a week and remains high

—though falling gradually—during early convalescence, then the presumption is strengthened. The criterion of a positive reaction is of course dependent on the behaviour of the strain of organisms employed for the test with sera from normal individuals and also from *cases of febrile illness due to other causes*. It is to be emphasised that satisfactory results are likely to be obtained only if those who employ the reaction have carefully determined this point for themselves. Not merely do different strains of the same organism show differences in their susceptibility to agglutination, but a given strain may vary from time to time ; this is the case with paratyphoid B bacilli, which may show spontaneous agglutination in the absence of serum ; to obviate this the recommendation has been made to emulsify paratyphoid B in 0·425 per cent. NaCl solution. Dreyer's formalinised broth cultures have the advantage of being standardised, since each batch is compared with a previous batch as regards its susceptibility to agglutination and by means of a factor the endeavour is made to render readings obtained with different batches strictly comparable. It is doubtful on general principles, however, whether a difference of as much as 100 per cent. in repeated tests has any definite significance. The standard suspensions require some special experience in their use, as they appear so much less dense than suspensions of agar cultures ordinarily employed for the sedimentation method. However, when the reaction is evident after exposure for two hours at 50°–55° C.

in the water bath, as recommended, very definite flocculation of the standard cultures can be seen; subsequently, after standing for a further period at room temperature the results may be less easily read (the use of the water bath is an objection to the method; the scum which is liable to form on the outside of the tubes must be carefully removed in order to observe the results). The sedimentation test is carried out as described above; the ultimate concentrations of serum in the mixture with bacterial suspension recommended by Dreyer are 1 : 25, 1 : 50, 1 : 125, 1 : 250. We prefer in general a geometrical series, viz. 1 : 50, 1 : 100, 1 : 200, 1 : 400, 1 : 800, 1 : 1600, 1 : 3200, etc. The reason for the use of this simple geometrical series is that errors are extremely unlikely to occur in the course of the manipulations involved in making the dilutions. As regards the preparation of dilutions, the use of graduated pipettes is advisable for many workers (see p. 52).

Procedure

Suppose that only 0·1 c.cm. of the patient's serum is available after separation from the clot, proceed as follows:

I. To a series of seven test-tubes ($3 \times \frac{1}{2}$ in.) add respectively, to the first 2·4 c.cm. saline and to each of the rest 1·0 c.cm. Then transfer 1·0 c.cm. from No. 1 to No. 2, mix well by drawing up and blowing out the mixture; then add 1·0 c.cm. of No. 2 to No. 3 and so on. The same pipette may be used throughout the series if care is taken that each mixture is drawn up and blown out several times.

II. Place in a rack three rows each of eight test-tubes ($3 \times \frac{1}{2}$ in.). Add in order to No. 8 of each row 0·3 c.cm. saline, then to No. 7 of each row 0·3 c.cm. of serum dilution (7) and so on, proceeding from No. 6 to No. 1, using the same pipette throughout.

- III. (a) Add to tubes 8, 7, 6-1 of the front row 0·3 c.cm. of *B. typhosus* emulsion (see p. 25).
- (b) " " " " mid row 0·3 c.cm. of *Para-typhosus* A.
- (c) " " " " back row 0·3 c.cm. of *Para-typhosus* B.

When Dreyer's emulsions are used, keep the tubes in the water bath at 50-55° C. for two hours and then after fifteen minutes further at room temperature take the reading. In the case of agar-culture suspensions, incubate at 37° C. for two hours, preferably removing the tubes from the incubator for ten minutes every half-hour. As in the case of testing unknown organisms with antisera, so here also the clearest results are obtained when sufficient time has been allowed for maximum agglutination to occur, *i.e.* by letting the tubes stand overnight at room temperature subsequent to the two hours' period of incubation at 37° C. This is well illustrated by the following case (Mackie and Wiltshire):

PATIENT INOCULATED WITH TYPHOID VACCINE ELEVEN MONTHS
PREVIOUSLY: SEVENTH DAY OF ILLNESS: *B. PARATYPHOSUS* A ISOLATED

	Dilutions of serum.				
	1: 50	1: 100	1: 200	1: 500	1: 1000
Two-hours' reading :					
<i>B. typhosus</i> .	+	—	—	—	—
<i>B. paratyphosus</i> A .	+++	+	—	—	—
<i>B. paratyphosus</i> B .	—	—	—	—	—
Twenty-four-hours' reading :					
<i>B. typhosus</i> .	+++	++	—	—	—
<i>B. paratyphosus</i> A .	+++	+++	+++	++	—
<i>B. paratyphosus</i> B .	++	—	—	—	—

In the case of the hanging-drop method, mixtures should also be made accurately with pipettes, then a loopful of each is examined in the form of a hanging drop; a rapid method well adapted to the performance of numbers of examinations is that of Garrow.

The criteria of a positive reaction are as follows—marked agglutination with average cultures by the sedimentation method in the case of persons not vaccinated against the organism in question.¹

Organism.	Serum dilution.
B. typhosus	1 : 100
B. paratyphosus A	1 : 50 (or even lower, 1 : 20)
B. paratyphosus B	1 : 200
B. dysenteriae Shiga	1 : 50
B. „ Flexner-Y	1 : 100

These results are significant when obtained at the end of the first week of fever in typhoid-group infections, but later on, in the second and third week, much higher dilutions cause agglutination, even 1 : 1000 or higher.

It is to be noted here again that these positive criteria are limiting values, *i.e.* the greater the dilution beyond these in which agglutination occurs, the more certain the conclusion that the agglutinat-

¹ Rajchman and Western (*Medical Research Committee, Special Report*, No. 5, 1917) have made a very extensive examination of the action of the sera of "normal" individuals on typhoid, paratyphoid, and dysentery bacilli, which served to control their tests in cases of bacillary enteritis. Unfortunately no details are given of the culture strains which they employed which would enable one to judge of their being of average character in the serological sense; hence their results lack the general application which they might otherwise have possessed.

ing action of the serum is due to a specific response to infection with the organism in question. Agglutination stopping at the limiting values is not to be regarded as even presumptively conclusive evidence, but merely as corroborative. In the case of Paratyphoid A infection the development of agglutinins is often especially poor at all stages, and Dreyer has concluded that agglutination with serum 1 : 10 is for all practical purposes diagnostic. The inference seems justified that the phenomenon of agglutination is often an unsatisfactory criterion of infection with this organism. *Each serum should, of course, be tested in a series of dilutions in order to determine the lowest concentration which causes agglutination.* Where there is the question of infection by any one of the typhoid-paratyphoid organisms, the serum should be tested with all three. The organism for which the agglutinin content of the serum is ultimately highest is in all likelihood the one responsible; as there is no evidence that infection with any one of the three organisms, *B. typhosus*, paratyphosus A or B, ever leads to more powerful agglutinins for either of the other two organisms of the group than for the one actually causing the disease, *i.e.* co-agglutination is not a disturbing factor. On the other hand, old observations have been recorded in which infections with meat-poisoning bacilli have led to higher agglutinating sera for *B. typhosus* than for the causal organism.

THE WIDAL REACTION IN INDIVIDUALS VACCINATED WITH TYPHOID BACILLI

B. typhosus.—The above-mentioned criterion of a positive Widal reaction for *B. typhosus* is, of course, quite inapplicable in cases which have been recently vaccinated with dead typhoid culture. Thus Martin and Upjohn found among seventy-five members of a hospital staff, who had not suffered from illness, but who had been inoculated with typhoid vaccine seven to fourteen months previously, that two-thirds agglutinated *B. typhosus* in a serum dilution of 1 : 200 or upwards, while a tenth agglutinated in 1 : 800¹; hence it is probable that *agglutination with a dilution of more than 1 : 1000 strongly suggests infection with B. typhosus in a person vaccinated with that organism six to eighteen months previously*. On the other hand, in an undoubted *B. typhosus* infection in an inoculated individual the agglutinin content of the serum may never reach a significant level, *e.g.* in a case which yielded *B. typhosus* in the urine, the highest agglutinin titre of whose serum was 1 : 200, reached at the end of the illness (Mackie and Wiltshire).

Paratyphoid Bacilli.—In individuals who have been vaccinated against *B. typhosus*, paratyphoid infections may be determined as described above; if the test is equivocal at an early period of the

¹ A sedimentation method was employed and the results were read after two hours at 37° C. and one hour further at room temperature.

illness a subsequent examination may afford conclusive proof, as the following example shows (Mackie and Wiltshire) :

CASE INOCULATED WITH TYPHOID VACCINE ELEVEN MONTHS PREVIOUSLY

	Serum dilutions.					
	1 : 50	1 : 100	1 : 200	1 : 500	1 : 1000	1 : 2000
Fourth or fifth day of illness:						
B. typhosus	+	—	—			
B. paratyphosus A . . .	+++	+	—			
B. paratyphosus B . . .	—	—	—			
Thirteenth day of illness:						
B. typhosus	+++	++	+	—	—	—
B. paratyphosus A . . .	++++	++++	++++	++++	+++	++
B. paratyphosus B . . .	++	—	—	—	—	—

Even in spite of the frequently poor agglutinin development in the serum in paratyphoid A infections, a considerable number can be diagnosed by the Widal reaction; thus, Mackie and Wiltshire found in a series of thirty-two cases from which B. paratyphosus A was isolated, that twenty-three showed a specific agglutinin effect, *i.e.* agglutination of this organism with higher dilutions of serum (1 : 50 and upward) than agglutinated either B. typhosus or B. paratyphosus B—the macroscopic method being employed with suspensions of living agar cultures.

“ Vaccine Effect ”

It is most important to note, however, that in one who has been vaccinated with dead B. typhosus

a subsequent infection, *e.g.* with paratyphoid bacilli, may cause also an increase in the typhoid agglutinins in the blood, which tend to appear earlier and to persist for a shorter time than the specific agglutinins for the infecting organism (Martin and Upjohn); further, the agglutinating power of the serum for *B. typhosus*, thus developed, may be greater than that for the infecting organism. Other infections, *i.e.* febrile conditions in general (Vincent), *e.g.* acute miliary tuberculosis, may produce the same effect, thus leading to an increase in typhoid agglutinins in the serum during the course of the disease. On the other hand, certain observers have recorded the sudden disappearance of agglutinins generated by antityphoid inoculation, subsequent to acute infections (Tidy). Mackie and Wiltshire have found that the typhoid agglutinins which are present during a paratyphoid infection in a person previously inoculated only with *B. typhosus* vaccine are true typhoid agglutinins, since they are absorbed from the serum by *B. typhosus* and not by paratyphoid bacilli.

METHOD OF ABSORPTION TESTS

Four c.cm. of a 1 : 25 dilution of serum divided into two equal parts. Two twenty-four hours' agar slope growths of *B. typhosus* were emulsified in one part, and two similar agar slopes of the particular paratyphoid organism in the other. The emulsions were incubated for three to four hours, at the end of which time the clear supernatant fluid (separated by means of the centrifuge) was carefully pipetted off from the bacillary sediment.

The usual series of dilutions was then made from each portion

of the serum and both were tested with *B. typhosus* and also with the paratyphoid organism in question. The following is an example of the results obtained:

READING AFTER TWO HOURS AT 37° C.

	Dilutions of serum.				
	1:50	1:100	1:200	1:500	1:1000
Untreated serum:					
<i>B. typhosus</i> . . .	++++	++++	++++	+++	—
<i>B. paratyphosus</i> A . .	—	—	—	—	—
<i>B. paratyphosus</i> B .	++++	++++	+++	++++	++
Serum absorbed with <i>B. typhosus</i> :					
<i>B. typhosus</i> . . .	—	—	—	—	—
<i>B. paratyphosus</i> A . .	—	—	—	—	—
<i>B. paratyphosus</i> B .	++++	++++	++++	+++	+
Serum absorbed with <i>B. paratyphosus</i> B:					
<i>B. typhosus</i> . . .	++++	++++	+++	++	—
<i>B. paratyphosus</i> A . .	—	—	—	—	—
<i>B. paratyphosus</i> B .	—	—	—	—	—

Thus it is clear that independent agglutinins to *B. typhosus* and *B. paratyphosus* B were present in the serum.

The Diagnosis of Mixed Infections by Agglutinin Absorption

The following result in a case showing moderately high titre agglutinins for both *B. paratyphosus* A and B—in the absence of isolation of the infecting organisms—points to mixed infection with both paratyphoid A and B bacilli, since independent agglutinins to both strains of bacilli were present, as well as to *B. typhosus*—the patient having been

inoculated with the last-named organism (observation of Mackie and Wiltshire).

Careful absorption tests were carried out, the serum being saturated with *B. typhosus*, paratyphosus A, and paratyphosus B., and the "absorbed" sera tested against these organisms. The results show the presence of independent agglutinins to all three strains of bacilli:

PATIENT INOCULATED WITH *B. TYPHOSUS* VACCINE TEN
MONTHS PREVIOUSLY

(Test about fourteenth day of illness)

	Serum dilutions.			
	1:50	1:100	1:200	1:500
Untreated serum:				
<i>B. typhosus</i> . . .	++++	++++	+	—
<i>B. paratyphosus</i> A . .	++++	++++	++++	++
<i>B. paratyphosus</i> B . .	++++	+++	++	+
Serum after absorption with <i>B. typhosus</i> :				
<i>B. typhosus</i> . . .	—	—	—	—
<i>B. paratyphosus</i> A . .	++++	++++	+++	+
<i>B. paratyphosus</i> B . .	++++	+++	+	—
Serum after absorption with paratyphosus A:				
<i>B. typhosus</i> . . .	++++	++++	—	—
<i>B. paratyphosus</i> A . .	—	—	—	—
<i>B. paratyphosus</i> B . .	++++	+++	+	—
Serum after absorption with <i>B. paratypho-</i> <i>sus</i> B:				
<i>B. typhosus</i> . . .	++++	+++	—	—
<i>B. paratyphosus</i> A . .	++++	++++	+++	+
<i>B. paratyphosus</i> B . .	—	—	—	—

Readings after two hours at 37° C.

Accordingly, the Castellani reaction (see p. 29) appears to have a valuable application in determining the infecting organism in such cases.

Significance of the Date of Maximum Agglutination

Dreyer and Inman conclude that the maximum agglutination titre for the infecting organism most frequently occurs about the eighteenth to the twentieth day and almost always lies between the limits sixteenth to twenty-fourth days; if this should prove to be the rule in an extensive series of cases controlled by bacteriological diagnosis in the form of repeated isolation of the same type of organism from blood and faeces or urine, it will afford a most valuable diagnostic phenomenon. It will be remarkable if this biological reaction is found to occur with such regularity as to time, although notoriously so irregular in the quantitative aspect, in a disease which is otherwise frequently so anomalous in its manifestations, and no effort should be spared to secure extensive verification.

The Widal Reaction in Triply Vaccinated Persons.—In those vaccinated against all three organisms of the typhoid group, especially if recently, it is doubtful whether any serological criteria will be capable of affording strong presumptive evidence as to the nature of the disease, even should the agglutinin content of the serum undergo a very marked increase for one organism alone in the course of the

illness. While remarkably interesting and suggestive work has been done on the curves of agglutinin content in the serum in cases of "enterica" which have undergone triple vaccination, it cannot be too strongly urged that the results are likely to continue to be equivocal in a considerable proportion; there has not yet appeared any conclusive bacteriological proof whereby to control the interpretation of agglutinin curves in triply inoculated patients; but the controversy which has attended this subject indicates that it is likely to remain for some time merely a matter for argument and that the individual curves in a considerable proportion of cases will lead to opposed interpretations. Frequently the method of diagnosis by agglutinin curves does not lead to results which permit of a convincing diagnosis, as when cases are examined late in convalescence (see Glynn and Lowe's series and Garrow's observations on these cases). So far, agglutination curves in cases which have been triply vaccinated do not by themselves appear to afford a reliable basis for any practical therapeutic or administrative measure. Thus if a choice has to be made between time spent on agglutination tests and on efforts to isolate the specific organism, the latter is much the more important object.

To this the objection may be raised that it is frequently impossible to secure the case sufficiently early to obtain a positive blood culture, and that the isolation of the specific organisms from faeces and urine is a matter of great uncertainty, thus

throwing one back on the Widal reaction with the patient's serum. We believe, however, that statistics such as the following of Priestley and Whitehead (unpublished) represent what can be accomplished : in 322 cases of “ enterica ” notified primarily on clinical grounds and in which no other infection could be determined bacteriologically the following results were obtained :

Bacteriological Diagnosis (isolation of organism).	Organism.			
	B. typhosus.	B. paratyphosus A.	B. paratyphosus B.	Total.
Blood culture	10	24	18	52
Urine	4	2	5	11
Faeces	5	15	41	61

No. of cases in which specific organisms were isolated . 124

Diagnosis established solely by serological method . 23

Provided the cases are examined within the first week, blood culture will be almost uniformly successful in leading to the detection of typhoid-group infections ; thus in a series of upwards of 130 cases of acute febrile illness Smith (unpublished) obtained thirty-four positive results, and of the remainder no single one was subsequently proved to be suffering from a typhoid-group infection in spite of extended bacteriological and serological investigations.

A further point of great practical importance in dealing with such infections, which may possibly assume epidemic character, is that frequently the serological test is not likely to yield even presump-

tive evidence as to the specific cause until a time which is probably much too late for any effective measures of prevention or treatment.

CARRIERS

The detection of carriers depends ultimately on the isolation of the specific organism from their tissues or excretions. This is a difficult problem, since neither the number of organisms excreted nor their periodicity can be foretold, and a case which is extremely difficult to discover by bacteriological examination may apparently have transmitted the disease extensively to others. The Widal reaction if positive in a suspected carrier strengthens the suspicion materially; on the other hand, carriers may manifest no serological reaction to distinguish them from normal individuals, and there is no procedure known which will replace the extended examination of faeces and urine in searching for carriers (see examination of faeces and urine, pp. 113, 133, 138). It is obvious that in view of the epidemiological importance of such persons, especially in a population unprotected by antityphoid vaccination, no pains should be spared to identify them and to prevent them from occupying positions in the distribution and handling of milk and other foodstuffs, where they could act as massive disseminators of the disease. A serious problem which remains to be solved is whether or not antityphoid vaccination, while undoubtedly protecting against serious manifestations as the result of infection, really increases

the number of those who become chronic carriers. The intimate mixing of a vaccinated and an unvaccinated population previously more or less separate, as when inoculated troops return to civil life, may have serious epidemiological results. The importance also of young children in spreading these infections, owing to the mild form in which the disease attacks them, must be borne in mind. It is difficult to decide for practical purposes how many examinations of the faeces should be performed during convalescence in order to determine that a case is unlikely to become a chronic carrier. Three negative consecutive weekly examinations have been widely accepted as a satisfactory measure, but this is purely arbitrary and no standard can be fixed. In women, who constitute the majority of carriers and who in civil life have the greatest opportunity of disseminating infection, especial attention should be paid to this matter.¹

BACILLARY DYSENTERY

Clinically dysentery is characterised by the presence of blood and mucus in the intestinal evacuations. Laboratory investigation has shown that this condition may be due to *entamoeba histolytica*, *lamblia intestinalis*, bacilli of the dysentery group, and also apparently to bacilli of the typhoid-paratyphoid-coli group, including *B. Morgan I*, and to *B. faecalis alkaligenes*, *B. pyocyaneus*, and strepto-

¹ Ledingham and Arkwright's *Carrier Problem* (London, 1912) is a storehouse of information on carriers.

cocci, either singly or in combination. The diagnosis of the cause, which is highly important for therapy, can only be arrived at by laboratory investigations. Unlike the typhoid group, the dysentery bacilli have little tendency to invade the blood, hence blood culture does not afford positive aid; their numbers in the faeces, while preponderant during the first two or three days of the acute attack, diminish rapidly thereafter, and they may cease to be found after the tenth day. The group of true dysentery bacilli is one possessing heterogeneous characters; and while in temperate climates the Shiga and the Flexner-Y forms are highly characteristic and constitute two distinct types which are fairly stable in their agglutination characters, there appears to be a multitude of allied organisms which may act as the cause of dysentery (see Martin and Williams). Thus in Thomson and Mackie's investigation B. Flexner-Y infections were found in 25 per cent. and atypical dysentery bacilli in other 26 per cent. of the cases (the percentage of cases in which no definite finding was recorded amounted only to 7). The fermentative characters of several atypical strains are shown in the table (p. 17). The pathogenic import of such organisms when found in large numbers in the faeces during an acute attack is highly significant. But whether pathogenic importance is to be attached to many varieties of non-lactose fermenting organisms obtained for the first time in convalescent cases is as yet undetermined; the

question remains to be settled whether all or any of these organisms represent specific types which are highly pathogenic and are transmitted from person to person, causing disease in a considerable proportion of those whose intestines they reach, or whether, on the other hand, they are organisms normally present in the intestine of the individual, which in ordinary circumstances are held in check by the common flora, but under conditions not yet understood are capable of replacing the usual coli-types. Morgan's observations on infantile diarrhoea and Thomson and Mackie's on dysentery in Egypt are extremely suggestive of the organisms, which they describe, being true pathogenic entities. It may be that some of the latter represent strains of bacilli to which the natives are more or less immune, but to which Europeans are not accommodated; the pathogenic results of intraperitoneal injections of such bacilli in the living state into animals are not very helpful in this respect. On the other hand, the number of varieties of coliform organisms present in normal faeces is remarkably large and when selective media are employed they can be brought into great prominence. The whole problem is a highly complicated one and no solution is yet possible; thus, few doubt the etiological relationship of *B. typhosus* to typhoid fever, and yet the demonstration of this organism in the faeces may be a matter of extreme difficulty or impossible, even in cases in which it can be isolated from the intestinal ulcers post mortem. Accordingly, neither the

prevalence nor the scantiness of any given organism in the intestinal contents affords definite evidence as to its pathogenic importance, and it is most probable that when once the specific organism has initiated lesions, the disease is intensified or perpetuated in the individual by bacteria which were incapable of starting the process. When an organism causes septicaemia or constantly localises in tissue lesions in practically pure culture, or when it causes such a reaction as to produce a marked degree of anti-substance content in the blood, then its claims to pathogenic significance are considerable (for the Widal reaction in bacillary dysentery, see p. 34). But in the case of many of the atypical organisms recovered late in the disease the evidence on these points is not yet full. McNee (unpublished) has isolated lactose-fermenting bacilli from dysenteric faeces, which are agglutinated to the limit by anti-dysentery-Ysera; this may be merely paragglutination (p. 173).

The tendency toward relapse shown by dysenteric infections is great, and the alternate appearance of the bacilli and their disappearance from the faeces is an important phenomenon as yet inexplicable. From the practical point of view the recognition of these cases in the late and non-acute stages is difficult or impossible owing to the scantiness of the specific organisms in the faeces, and no selective antiseptic is so far known which may be employed for an enrichment medium.

ADDENDUM

MARRIS'S ATROPINE TEST FOR TYPHOID-GROUP INFECTIONS¹

THE time chosen for the test should be at least one hour after a meal and the patient must be kept perfectly quiet and in the horizontal position. The pulse is recorded minute by minute until it is found to be steady—after ten minutes usually; then $\frac{1}{33}$ grain atropine sulphate is injected hypodermically in the triceps region. After an interval of twenty-five minutes the pulse is recorded again, minute by minute (for fifteen to twenty minutes) until it is clear that any acceleration which has followed the atropine is passing off. If the maximum rate after the injection exceeds the mean rate beforehand by twenty beats or more per minute (any extraneous cause of acceleration, such as muscular movement, coughing, etc., being excluded), then this may be accepted as an indication that the patient is not suffering from typhoid or paratyphoid infection. *If the maximum increase after injection is ten or less, this is in favour of the case being one of typhoid or paratyphoid infection.* An intermediate increase may be regarded as more doubtful (although the statistics indicate that fourteen is the critical number), and the test should then be repeated several days later; three negative reactions within the first fortnight of illness exclude the presence of typhoid with a considerable degree of certainty. The reaction is most constantly

¹ Marris points out that the data were obtained in the case of young men on military service.

obtained in the second week of the disease, especially on the tenth day. After the fourteenth day of illness positive reactions are alone significant; but of 111 typhoid-paratyphoid-group infections from which the specific bacteria were isolated 104 reacted positively to the atropine test, *i.e.* 94 per cent.—15 of the 104 cases, however, showed a rise of 11-14 beats per minute (see *Medical Research Committee's Report*).

The test is unreliable in individuals over fifty years of age, as atropine then causes less acceleration normally; also those of any age who suffer from arteriosclerosis fail in general to give the acceleration response, and when the heart is already rapid (100 or more) and irregular the test is unreliable. An extensive series of other acute infections—proved by bacteriological examination—all showed the acceleration: *viz.* dysentery, bacillary and amoebic; cerebro-spinal fever; tuberculosis, meningeal, pulmonary, and miliary; septic meningitis; malaria; relapsing fever; jaundice, exclusive of that associated with typhoid or paratyphoid infections; B. Gaertner infection; septicaemia; also clinical cases of influenza, etc. It is important that typhoid (two cases) and paratyphoid (four cases) carriers all failed to give the reaction, *i.e.* they behaved like normal individuals.

Repetition of the atropine does not alter the reaction.

Inoculation with typhoid-group vaccines causes a positive reaction which sets in after one to three days, but may be delayed (to the seventh day in one case). "So far as can be judged, the positive test to atropine which follows inoculation may last some four or five weeks." Inoculation with cholera vaccine (two cases) and vaccinia lymph (three cases) in no instance led to a positive reaction.

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III

THE USE OF CALIBRATED PIPETTES IN SEROLOGICAL WORK

BY C. H. BROWNING

THE following is a description of the method of quantitative operation in general serological work which has been practised in the Pathological Department of the University and Western Infirmary, Glasgow, for over fifteen years. Two sizes of pipette¹ are employed: (1) 0.1 c.cm. pipettes of thermometer tubing; external diameter $\frac{11}{64}$ in.; the length of the column of fluid corresponding to 0.1 c.cm. is $6\frac{1}{8}$ in. The pipette tapers for the last $\frac{3}{4}$ in. and the external diameter at the orifice is $\frac{3}{64}$ in. They are graduated to 0.002 c.cm. (2) 1 c.cm. pipettes; external diameter $\frac{1}{4}$ in.; length of column of fluid corresponding to 1 c.cm. = $8\frac{1}{2}$ in.; external diameter at orifice $\frac{1}{16}$ in.; they are graduated to 0.01 c.cm. Both kinds of pipettes are graduated to the orifice.

The 1.0 c.cm. pipette is used for amounts greater than 0.1 c.cm. and quantities differing by 0.05 c.cm. can be accurately measured; the 0.1 c.cm. pipette

¹ The pipettes can be obtained from Messrs. Hawksley & Sons, 357 Oxford Street, London, W.1.

1871

1872

1873



FIG. 1.

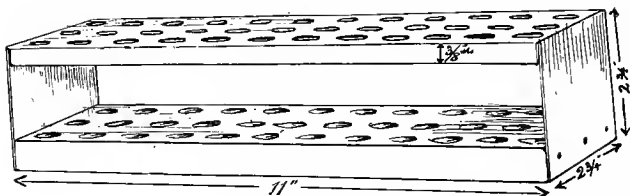


FIG. 2.

[To face page 53]

is for smaller amounts and quantities differing by 0.005 c.cm. can be accurately delivered.

For use the pipette may be actuated by an indiarubber teat, but we greatly prefer a mouthpiece of plain glass quill tube (No. 5), the free end being cut across transversely and smoothed in the Bunsen flame and the other end slightly drawn out so as to slip readily on to a convenient length of not too slender indiarubber tubing to which the pipette is attached. The mouthpiece method is the more rapid, and accuracy is more readily attained by its use in our experience. To obviate the danger of sucking up living cultures into the mouth a small piece of cotton wool may be packed loosely into one end of the indiarubber tube, but this should be changed when it becomes moist. When the pipette is being used the mouthpiece is held between the teeth and the junction between the glass stem of the pipette and the attached indiarubber tube is held between the thumb and first and second fingers of the right hand. In order to draw up a measured amount, say 0.05 c.cm., into the fine pipette—(1) draw up fluid to slightly above the 0.05 mark, then apply the tip of the tongue to the orifice of the mouthpiece, so as to close it, and continue to keep the tongue in this position until operation (4) is completed; (2) withdraw the nozzle of the pipette from the fluid, and bring it against the side of the vessel *above* the level of the fluid; (3) while maintaining the nozzle continuously against the vessel wall, as described under (2), make slight pressure on the rubber with the fingers and thumb of the right hand until the level of the fluid in the pipette reaches the 0.05 mark; then cease making any further pressure; (4) insert the pipette into the vessel into which the fluid is to be delivered (if the amount to be delivered is very small, *e.g.* 0.01 c.cm., pass the nozzle down to within a short distance of the fluid in the tube) and (5) blow out vigorously—while blowing, bring the nozzle into contact with the side of the tube above the level of the contents. (The object of bringing the nozzle against the vessel wall in (3) and (5) is to ensure that the fluid shall drain away from the pipette and not remain at the nozzle as a drop which would cause a serious error in the measurement of the amount delivered.) The illustration (Fig. 1) shows the mode of holding the pipette, the reader being in the same position as the operator.

It is difficult to understand why for most purposes involving the measurement of small quantities of watery fluids these pipettes should not be widely employed, since they are cheap and readily replaced and also measurements made with different pipettes correspond with a sufficient degree of accuracy. It is, of course, clearly understood that the amount ejected does not correspond precisely with the quantity indicated on the graduated stem, but this error occurs throughout, hence comparable results are obtained. When it is required to measure out considerable quantities (0.25 c.cm., *i.e.* about four drops from the 1 c.cm. pipette, and upward) the graduated pipette is more rapid than a drop method; for many workers it is also certain to be much more accurate, since, from the experience of ourselves as well as others, we could not recommend the procedure of counting fifty-seven drops! Also, the capillary pipette with a "fiducial mark" into which successive columns of fluid are drawn up, each separated by a minute air bubble, is a most ingenious piece of apparatus; but while specially suited for the manipulations of those who estimate the opsonic index, it is not adapted for the rapid measurement of varying and considerable amounts of fluid, such as are required in accurate general serological work. Large numbers of pipettes need not be employed, several suffice for most purposes, and they need not be dried between successive operations. They should be well rinsed by drawing up and rejecting 0.85 per cent. saline between each operation; and

where there is danger that a trace of any reagent might have a powerful action, *e.g.* a powerful agglutinating antiserum in agglutination experiments, the pipette, after its albuminous contents have been well washed out, should be boiled for a minute or so in the sterilising bath and *then allowed to cool thoroughly before further use.* (Great trouble may be caused by allowing serum to evaporate or coagulate in the pipettes; they are then best cleared by passing fine wire through the nozzle and along the stem; in addition, they may be soaked in nitric acid.)

STORAGE OF ANTISERA

Pieces of quill tube (of about 1.5 c.cm. capacity) are drawn out to capillary ends, each about one inch long and are sterilised by dry heat. Each is filled by inserting one end into the indiarubber tube attached to the mouthpiece above described, then it is filled like a pipette, but during the filling the fingers maintain a slight degree of pressure on the indiarubber tube. When the quill tube is about three-quarters full the free end is removed from the vessel of serum, and then the pressure of the fingers on the indiarubber is released—the tongue keeping the end of the mouthpiece closed throughout. The result is that a small amount of air enters the free end of the tube, which can then be sealed in the flame; thereafter the other end is sealed. One advantage of this method of storage is that a small amount of serum can readily be withdrawn as required.

FORM OF RACK EMPLOYED IN SEROLOGICAL WORK

(SEE ILLUSTRATION—FIG. 2)

Specification

Top and sides are formed by a single piece of No. 22 B.W. gauge zinc; perforations in the top are each $\frac{9}{16}$ in. in diameter. Base of hard wood $\frac{7}{8}$ in. thick, in which are cupped depressions $\frac{1}{4}$ in. deep in the centre, and $\frac{9}{16}$ in. in diameter.

Other measurements are shown.

IV

OBSERVATIONS ON THE DIPHTHERIA GROUP WITH SPECIAL REFERENCE TO THE RECOGNITION OF PATHOGENIC MEMBERS

BY J. F. SMITH, GLASGOW

THE diagnosis of diphtheria is an important problem. As a rough practical measure in patients who are clinically suspicious, the morphological characters and staining reactions of an organism in cultures from the throat are generally taken as distinctive ; but when, especially, the clinical features are indefinite or the organism is derived from a site other than the throat, *e.g.* so closely neighbouring a part as the nose, the determination is much more difficult. In the course of investigations on ozoena about fifty strains of organisms of human origin, more or less closely resembling *B. diphtheriae*, have been examined, including twenty-three strains of virulent diphtheria bacilli and many cultures of diphtheroid bacilli from the nose ; the following observations have been recorded, since, although no claim to originality is made for them, nevertheless they are not generally mentioned in textbooks.

STAINING

The presence of granules which stain deeply with Loeffler's methylene blue in organisms from cultures on serum, does not necessarily mean that the Neisser reaction will be positive. Thus, Neisser-negative cultures of diphtheroid bacilli have been met with, which showed marked, deeply staining terminal granules in films treated with methylene blue.

The appearance of Neisser-positive granules depends on several factors which vary with different strains. Thus, with one strain of *B. diphtheriae* which gave positive Neisser staining after rough fixation in the flame, the result was negative after fixation with Flemming's solution and with 5 per cent. formalin in saline ; on the other hand, another strain showed characteristic granules after similar treatment in the case of both these fixatives. Good staining of the granules was in general obtained after exposure of films to 120° C. for twenty minutes. It has been found that some strains failed to show Neisser granules when the preparations were washed in hard water, *e.g.* London tap water, in the course of staining, whereas the same cultures showed characteristically stained granules when distilled water was employed. This irregularity in staining when tap water is used to wash the preparations may explain the disfavour into which the Neisser reaction has fallen with a number of workers.

CAN TRUE VIRULENT *B. DIPHTHERIAE* BE DISTINGUISHED BY FERMENTATION REACTIONS ?

Were it possible to identify true *B. diphtheriae* by means of fermentation reactions a great simplification in diagnosis would be effected, and Hine has suggested that this might be the case. Thus he says: "I have yet to find an organism of the diphtheria group, giving acid with glucose and dextrin, but not with saccharose, which proves to be other than the *B. diphtheriae*; all of the organisms giving this reaction were fully virulent. I, therefore, consider that in this test we have a valuable means of distinguishing between the *B. diphtheriae* and other allied organisms, and one which will help observers who are not in a position to submit a doubtful bacillus to an animal experiment."

The following observations bear directly on this point :

Glucose was fermented with acid formation by all virulent and otherwise typical bacilli, generally in twenty-four hours ; but many non-virulent, and one of two virulent, but atypical strains, also fermented glucose rapidly.

Saccharose was not fermented by any strain otherwise typical ; but an atypical virulent strain (the one which fermented glucose) formed acid from this sugar in twenty-four hours.

Dextrin was fermented by most of the organisms tested ; but three virulent and otherwise

typical strains failed to alter dextrin, as also did a number of atypical non-virulent bacilli.

Lactose was fermented only by two organisms; one was an atypical, virulent strain, the other was an atypical, non-virulent organism from the nose, in a case of atrophic rhinitis.

Maltose was fermented by all virulent organisms tested, except one. This bacillus was obtained from the throat of a patient clinically suffering from diphtheria; it appeared morphologically to be *B. diphtheriae*, and caused the death of a guinea-pig within twenty-four hours, the heart-blood being sterile.

Diphtheroid bacilli from crusts in atrophic rhinitis (for the isolation of such organisms, see p. 146).—Seven strains fermented glucose as readily as did true *B. diphtheriae*; a number of these were indistinguishable morphologically and in staining reactions from true diphtheria bacilli, and also fermented dextrin, but not saccharose; all were non-virulent. Since similar organisms appear to inhabit the nose in conditions other than atrophic rhinitis and are more prevalent there than in the throat, the important question arises, Where does the nasal region cease and the throat commence as regards the habitat of these organisms?

Atypical but virulent strains of diphtheroid bacilli.—Two such cultures were isolated from throat swabs in cases clinically suggesting diphtheria; neither gave positive Neisser staining in twenty-four-hour

cultures on Loeffler's serum, but both showed marked beading with methylene blue. One formed acid from glucose, saccharose, maltose, and lactose. The other rendered glucose alkaline in twenty-four hours, but formed acid from dextrin and maltose. In each instance 0.5 c.cm. of a forty-eight-hour serum-bouillon culture, injected subcutaneously, killed guinea-pigs in thirty-six hours; but the organisms were recovered from the heart-blood.

Thus it can be said that an organism which fails to ferment glucose is not a true diphtheria bacillus, but it may be highly virulent and pathogenic. On the other hand, *no combination of fermentative characters can be made the criterion of a virulent B. diphtheriae*. This conclusion is in accord with the recent results of Cary.

THE IMPORTANCE OF OBTAINING PURE CULTURES OF B. DIPHTHERIAE

Experience has shown that it is frequently extremely difficult to obtain pure cultures of diphtheria bacilli. When isolated colonies are subcultured it is often found that streptococci are present. Thus, in order to make sure of obtaining pure growths repeated plating should be performed. It is suggested that discrepancies in the results of different workers may be due to inattention to this point in some cases; the value of telluric-acid serum agar for this purpose is discussed on p. 146.

THE FORMATION OF INVOLUTION FORMS

It has been found that the addition of salicylic acid (0.004 per cent.) to peptone-water agar prevented the development of involution forms in five days (at 37° C.) by a strain which on the same agar, without salicylic acid, developed marked involution appearances in twenty-four hours. The inhibition produced by the salicylic acid was manifested by the development of small colonies at first ; but the individual colonies attained in five days to a greater size than those of the control of twenty-four hours.

CONCLUSIONS

Not merely are the relationships of virulent, toxin-producing and avirulent or non-toxic diphtheria bacilli and diphtheroids of great theoretical interest, but for the administrative control of diphtheria it is highly important that the carriers of organisms likely to give rise to cases of diphtheria should be efficiently dealt with. Thus time and expense ought not to be wasted in connection with those who harbour organisms devoid of pathogenic significance. How is such a distinction to be effected? Probably the best solution of the problem at present feasible is to investigate the pathogenic properties of the organism in question by injecting fluid cultures into guinea-pigs—the most economical procedure of this kind being the intracutaneous method (see Zingher and Soletsky). Recent investigations

have indicated that virulence and toxin production are not necessarily parallel properties (Cary), but it is probable that by estimating the effect of fluid cultures a significant resultant value of these attributes is obtained. Since it has up till now proved impossible to demonstrate alterations in the pathogenic properties of *B. diphtheriae* either by mutation or by any special procedure, and because there is no evidence either experimental or clinical which indicates that an avirulent, non-toxic strain of *B. diphtheriae* can become pathogenic, there is, therefore, at the present time, no valid reason for singling out in any way the carriers of such non-virulent and non-toxic organisms for isolation or treatment.¹ At the same time, it cannot be said that finality has been reached as to the permanent distinction between pathogenic and non-pathogenic strains.

To what process of sorting should strains be subjected before submitting them to the animal test? No organism which produces true diphtheria toxin has been found incapable of forming acid from glucose; hence the preliminary test on glucose is of value, since all non-glucose fermenters can be excluded. *But no further fermentation test and no combination of tests will satisfactorily distinguish the virulent and the toxin-producing organisms from all the others.* On the other hand, non-fermenters of glucose obtained from throat cultures, although not true *B. diphtheriae*, may possess high virulence for

¹ In regard to the treatment of diphtheria carriers, see p. 87.

animals, and the question as to whether these may not be important pathogenic factors in certain cases remains still to be investigated.

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V

STUDIES ON ANTISEPTICS

RELATIONSHIPS BETWEEN BACTERICIDAL ACTION
AND CHEMICAL CONSTITUTION, WITH SPECIAL
REFERENCE TO SELECTIVE INHIBITORY ACTION
ON DIFFERENT SPECIES OF PATHOGENIC OR-
GANISMS: THE INFLUENCE OF SERUM ON
ANTISEPTIC ACTION: THE TIME RATE OF
LETHAL ACTION: THE EFFECT OF VARIATIONS
IN THE NUMBER OF ORGANISMS: BACTERIO-
STATIC ACTION: PRACTICAL AND THEORETICAL
BEARINGS OF THE INVESTIGATIONS

BY C. H. BROWNING, W. GILMOUR, AND
R. GULBRANSEN

(*From the Pathological Department of the University and Western
Infirmary, Glasgow, and the Bland-Sutton Institute of Path-
ology, Middlesex Hospital, London.*)

Papers incorporated

Journal of Pathology and Bacteriology, vol. xviii. 1913, p. 144.

British Medical Journal, 1917, i. p. 73.

British Medical Journal, 1917, ii. p. 70.

A CONSIDERABLE number of organic and inorganic
compounds have been examined for bactericidal

action toward different species of bacteria, with a view to discovering: (a) substances possessing specific bactericidal properties for particular organisms; (b) relationships between chemical constitution and bactericidal action. The methods employed were: (1) to incorporate varying quantities of a solution of the substance to be examined in peptone water agar neutral to litmus; stroke cultures were then made with dilute suspensions of twenty-four-hour growths of various organisms and were incubated at 37° C. for forty-eight hours or longer; (2) varying amounts of the substance to be tested, in a volume usually not exceeding 0.1 c.cm., were added to 1 c.cm. of the culture medium, which consisted in one series of 0.7 per cent. peptone water, and in the other of undiluted serum, and then immediately 0.1 c.cm. of a 1 : 20,000 dilution in saline of a twenty-four-hour peptone water culture was added.¹ A control was made with peptone water or serum without antiseptic; one loopful of this inoculated mixture when streaked immediately on agar yielded twenty or more colonies of staphylococcus or *B. coli* (the number of

¹ Dakin and Dunham have drawn attention to the possibility of error arising from decomposition of the antiseptic when substances such as the unstable chlorine compounds are added to the protein solution and an interval is then allowed to elapse before the organisms are added subsequently. It is to be noted, however, that control experiments have shown that the brief interval which elapsed in our experiments did not introduce this source of error, since tests carried out with chloramine-T in the order suggested by Dakin and Dunham yielded precisely the same result.

organisms present enabled subsequent increase or decrease to be conveniently determined, *e.g.* in timed experiments, see p. 78). The tubes were then placed at 37° C., and were examined at the end of twenty-four to forty-eight hours in order to determine the concentration of antiseptic which killed the organisms introduced; for this purpose subcultures were made on agar and also in peptone water.

BENZOL DERIVATIVES—PRINCIPALLY THE SALTS OF ORGANIC BASES

Special attention has been directed to the differences in action exhibited by analogous compounds belonging to the same series, but differing in the radicals substituted in side-chains, *e.g.* in the diamino-triphenylmethane group, the unsubstituted compound (Doebner's violet), the tetramethyldiamino substitution product (malachite green), and the similar tetraethyl derivative (brilliant green).

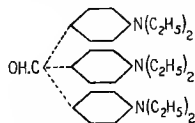
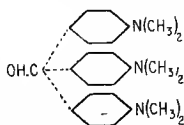
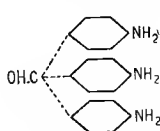
Certain general principles have been established: (1) *Staphylococcus aureus* and *B. anthracis* are more susceptible to the action of basic benzol derivatives than are organisms of the coli-typhoid group. This result has been obtained with **triphenylmethane** derivatives (*triaminotriphenylmethane* compounds—parafuchsin, fuchsin, hexamethyl-violet and hexaethyl-violet, methyl-green and the naphthalin compounds—victoria blue, new victoria blue, night blue; *diaminotriphenylmethane* compounds—Doebner's violet, malachite green, brilliant green, glacier

blue, setoglaucin, setocyanin); **acridine compounds** (3'6-diaminoacridine sulphate and chloride, acridine yellow, and other diaminoacridine derivatives containing methyl groups substituted either in the benzol rings or in the amino groups, also 3'6-diamino-10-methyl-acridinium chloride); **indamines** (Bindschedler's green); **azine dyes** (toluylene-red, safranin); the **thiazine group** (methylene-blue, new methylene-blue); thiopyronin. This is also the case with Ehrlich and Bechhold's antiseptics, tetrachlor-ortho-biphenol and tetrabrom-*a*-kresol. (2) It is not possible to differentiate generally the Gram-positive organisms as susceptible to hexamethyl-violet and the Gram-negative group as insusceptible; thus, *V. cholerae*, *B. cholerae gallinarum*, and *B. faecalis alkaliganes* were found to be all comparatively non-resistant to this dye.

RELATIONS BETWEEN CHEMICAL CONSTITUTION AND BACTERICIDAL ACTION OF ORGANIC COMPOUNDS

Triamino-triphenylmethane Group.—The unsubstituted triamino-triphenylmethane compound, para-fuchsin, has only a comparatively weak bactericidal action. The substitution of alkyl groups in the amino-side chains (hexamethyl-violet and hexaethyl-violet) leads to the most active bactericidal substances known for staphylococcus, *B. anthracis*, and *B. diphtheriae*; thus complete inhibition of growth of staphylococcus occurs with a concentration of 1 to 5 million to 1 in 1 million (see Table I)—the concentration of mercuric chloride required to produce

the same effect being 1 in 1 million to 1 in 250,000. The amount of parafuchsin and of hexamethyl-violet and hexaethyl-violet required to inhibit organisms of the coli-typhoid group is very much greater than that which inhibits staphylococcus—*B. typhosus* and *B. coli* being about equally susceptible; in the case of hexamethyl-violet ("crystal



Parafuchsin (carbinol) Hexamethyl-violet (carbinol) Hexaethyl-violet (carbinol)

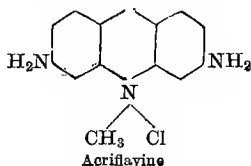
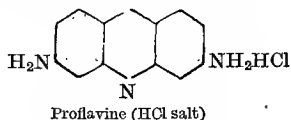
violet") in peptone water the ratio is 500 to 1. It is interesting to note that the addition of ethyl bromide to one of the amino groups of hexamethyl-violet, which produces the compound methyl-green, greatly reduces the bactericidal power for staphylococcus and *B. anthracis*.

Diamino-triphenylmethane Group.—The unsubstituted diamino-triphenylmethane derivative, Doebner's violet, has only a weak bactericidal action, which is more marked for staphylococcus and *B. anthracis* than for the coli-typhoid group; *B. typhosus* is not less susceptible than *B. coli* (see p. 100). The substitution of the four hydrogen atoms of the amino-radicals by methyl groups (malachite green) and ethyl groups (brilliant green) yields substances which possess the same order of bactericidal potency towards staphylococcus and *B. anthracis* as hexamethyl and ethyl violet; at the same time bril-

brilliant green is from five to ten times more actively bactericidal towards *B. coli* than malachite green and about twenty times more lethal for this organism than are the ethylated and methylated triamino-triphenyl-methane derivatives the (chemical formulæ are shown on p. 98). An important characteristic of brilliant green is that it exerts a more marked bactericidal action on the common types of *B. coli* than on *B. typhosus* or the paratyphoid bacilli; practical use has been made of this property for the isolation of these organisms from faeces (see p. 113). This difference between *B. coli* and *B. typhosus* is not observed in the same marked degree with malachite green and still less with propyl green (see p. 100). The addition of acid (hydrochloric or picric acid) in suitable amount abolishes the selective bactericidal action of brilliant green on *B. coli* (see p. 104), but the selective action is shown by the zinc double salt of brilliant green.

Acridine Group.—The substitution of one or more methyl groups, either in the benzol rings or in the amino-radicals or in both positions in 3·6-diamino-acridine (“*proflavine*”) reduces the bactericidal effect in watery medium; so that the diamino-acridine salts and diamino-methyl-acridinium chloride are more active than acridine yellow, etc.; *B. typhosus* is more susceptible to these substances than *B. coli*. Thus in the case of diamino-methylacridinium chloride (“*acriflavine*”) the lethal concentration for *B. coli* is ten times greater than that for *B. typhosus*. The effect of a great increase in the

number of organisms introduced into the antiseptic solution is to produce only a slight difference in the lethal concentration (see p. 82).



THE HEAVY METALS AND METALLOIDS AND RARE EARTHS

Heavy Metals.—In the case of these, most interesting results have been obtained. Contrasting with the general finding with basic organic derivatives, it appears that the salts of the heavy metals, mercury, silver, and gold, do not act more intensely on Gram-positive organisms, such as staphylococcus, than on *B. coli*. This is most marked in the case of silver nitrate, to which staphylococcus is more resistant than are many varieties of Gram-negative bacilli, as Table II shows (48 hours' incubation at 37° C.) ; the results with the same cultures tested simultaneously with proflavine are added by way of contrast (unpublished results of W. Gilmour):

Telluric acid is not a highly potent antiseptic,

ANTISEPTIC.

	Staphylococcus aureus.				Bacillus coli communis.			
	In Water + 0·7 per cent. Peptone.		In Serum.		In Water + 0·7 per cent. Peptone.		In Serum.	
	Lethal Concentration.	Antiseptic Potency.	Lethal Concentration.	Antiseptic Potency.	Lethal Concentration.	Antiseptic Potency.	Lethal Concentration.	Antiseptic Potency.
Carbolic Acid	1:250	1	1:250	1	1:500	2	1:500	2
Mercury Perchloride	1:1,000,000	4000	1:10,000	40	1:1,000,000	4000	1:10,000	40
Iodine (in KI) †	1:10,000	40	1:700	2·8	1:5000	20	1:800	3·2
Chlorine Water. Concentration of Chlorine .	1:2500	10	(1:1000 not sufficient)	—	1:2500	10	(1:1000 not sufficient)	—
Hypochlorites:								
“Eusol” (content in solution of 0·34 per cent.)	1:13	0·05	1:3·25	0·0125	1:13	0·05	1:3·25	0·0125
“Dakin’s Solution” (modified by Dauterive’s). (Content in “available” Cl = 0·22 per cent.)	1:4000	16	1:1000	4	1:4000	16	1:1000	4
“Chloramine-T” (content in “available” Cl = 25 per cent.)	1:9	0·036	1:2·25	0·01	1:9	0·036	1:2·25	0·01
Parafuchsin (Chloride)	1:4000	16	1:1000	4	1:4000	16	1:1000	4
Hexamethyl (Crystal Violet (Chloride) Malachite Green, Oxalate and Sulphate Brilliant Green Sulphate Brilliant Green Oxalate Ethyl-hydrocuprein Hydrochloride Acridavine (Diamino - methyl - acridinium chloride) Proflavine (Diamino - acridine sulphate or chloride)	1:20000	80	1:200,000	800	1:1300	5·2	1:100,000	400

Lethal Concentration = that which just suffices to kill organisms—as tested by subculture.

Antiseptic Potency = Ratio Lethal Concentration of Substance in question.

* Ox serum heated at 57° C. for half an hour was employed.

† Potassium iodide in the concentration employed in the solution of iodine by itself produced no bactericidal action.

TABLE II

	Organism.				
	Staphylococcus aureus.	B. coll.	B. Paratyphosus (A).	B. Paratyphosus (B).	B. Pyocyaneus.
5 c.cm. peptone-water agar, neutral to litmus, containing of AgNO_3 1: 1000					
0.05 c.cm.	abundant growth	abundant growth	abundant growth	abundant growth	abundant growth
0.1 c.cm.	" "	—	marked inhibition	marked inhibition	—
0.15 c.cm.	" "	—	—	" "	—
0.2 c.cm.	almost complete inhibition	—	—	—	—
Ditto, containing of Proflavine 1: 1000					
0.01 c.cm.	abundant growth	abundant growth	abundant growth	abundant growth	abundant growth
0.05 c.cm.	distinct inhibition	" "	" "	" "	" "
0.1 c.cm.	almost complete inhibition	" "	" "	" "	" "
0.2 c.cm.	—	distinct inhibition	" "	" "	" "

— = no growth.

but it has the almost unique property of being less inhibitory to the growth of *B. diphtheriae* and diphtheroid bacilli than to most other organisms except some streptococci; whereas the diphtheroid group is in general among the most susceptible to all other antiseptics so far investigated. This selective resistance of *B. diphtheriae* and diphtheroid bacilli was demonstrated by Smith; Conradi and Troch had previously employed a tellurate medium for identifying *B. diphtheriae* in mixed cultures; they, however, did not succeed in making the best use of this property for facilitating its isolation. J. F. Smith has devised an exceedingly simple and effective selective medium containing serum and telluric acid, which, so far as we know, affords the best conditions for recovering *B. diphtheriae* from mixed infections (see p. 141). Telluric acid has also been found by Browning, Mackie, and Smith to possess the remarkable characteristic of acting most strongly on many varieties of the coli group which present the greatest resistance to brilliant green (see p. 108). Hence a mixture of these two reagents is of value in the isolation of *B. typhosus*, where brilliant green alone does not yield positive results (see pp. 110, 119).

Salts of the Rare Earths.—A number have been tested, comprising caesium, cerium, didymium, erbium, lanthanum, rubidium, thallium, thorium, and titanium. None of these exhibits powerful antiseptic properties, but salts of thallium (acetate) and thorium (nitrate) show selective effects which

should prove most valuable for the isolation of certain organisms, since thallium suppresses the otherwise most resistant proteus types, while thorium permits the growth of *B. mallei* in concentrations which inhibit many other organisms (see p. 147).

HALOGENS

Under the conditions of experiment observed in these tests free *chlorine*, *hypochlorites* and *chloramines* (chloramine-T = sodium-toluene-para-sulphochloramide) are comparatively weak antiseptics. This is to be explained by the fact that these substances have very little selective affinity for the organisms in contrast to other organic matter. In the total absence of extraneous organic material, *e.g.* when acting on organisms suspended in pure water, these bodies are among the most powerful and rapidly acting germicides known. The results obtained with chloramine-T indicate a much weaker action in watery medium than that found by Dakin, Cohen, Daufresne, and Kenyon; the explanation of this lies in the fact that the medium used by us contained a small amount of peptone. We adopted this procedure advisedly, since we found, by plating, that in the absence of peptone a proportion even of staphylococci might cease to be viable when suspended in salt solution or water for several hours. In control experiments with *Staphylococcus aureus* suspended in water we obtained precisely the same results as Dakin, Cohen, Daufresne, and Kenyon—namely, sterilisation after exposure for two hours

to chloramine-T in 1 : 500,000 and survival in 1 : 1,000,000. In serum, also, we have obtained a much lower figure than Dakin, which is due to our tests being performed in a medium containing over 80 per cent. of serum, whereas Dakin used 50 per cent. serum (see also footnote, p. 66).

In the case of chlorine in distilled water contaminated with a suspension of an agar culture of *B. typhosus*, a concentration of 1 : 700,000 will produce complete sterility within five minutes, *provided that the number of organisms is suitably chosen*; with ten times as many organisms, forty-five minutes were required to attain the same result, as the following experiment shows (unpublished observation of Browning and Kennaway); care was taken that identical amounts of organisms were employed to make the subcultures in each case.

Example.—In each of two flasks 300 c.cm. distilled water containing chlorine 1 : 700,000—to A a watery suspension of *B. typhosus* equivalent to one-tenth of a 24-hour agar culture, to B the equivalent of a whole culture, were added and allowed to stand at room temperature. At intervals 1 c.cm. was withdrawn and at once neutralised by adding 1 c.cm. of n/1000 sodium thio-sulphate; of the samples from A, 0·2 c.cm. was plated in agar; from B, 0·02 c.cm. As controls similar amounts of *B. typhosus* in water containing no chlorine were plated after standing for 40 minutes.

Sample withdrawn after contact for	Resulting growth in	
	A	B
5 minutes	sterile	many thousand colonies
15 "	"	" " "
30 "	"	" hundred "
45 "	"	sterile
Controls	∞	∞

Iodine is more active than chlorine in the presence of dilute peptone water; but it is not a specially potent antiseptic and suffers great diminution in its activity in serum (see below).

ETHYL-HYDROCUPREIN

The hydrochloride of ethyl-hydrocuprein, introduced as a therapeutic agent for pneumococcus infections by Morgenroth and Levy, has been found by Wright to be powerfully bactericidal for pneumococci *in vitro*. On the organisms here investigated its lethal action is weak, but in the case of these, as Wright also found with pneumococcus, serum does not diminish its effect.

Table I shows the results obtained with the principal substances described above.

THE INFLUENCE OF SERUM ON ANTISEPTIC ACTION

The effect of serum on representative types of chemical bactericides is shown in Table I. Serum diminishes greatly the action of such powerful disinfectants as mercuric chloride, chlorine, chloramines, and also of many organic antiseptics, such as tetrachlor-ortho-biphenol. Phenol, although a weak antiseptic, is not reduced in potency by serum. In comparative experiments, in which the solution of antiseptic was added to a medium consisting of double-strength agar diluted with an equal volume of

serum (from the sheep, heated at 57° C., also unheated), or—in the case of fluid medium—in ox, rabbit, and human sera, heated at 57° C. or unheated, it was found that the presence of serum diminished the antiseptic action for staphylococcus comparatively slightly in the case of hexamethyl violet, to a considerable degree with brilliant green, and more markedly with tetrachlor-ortho-biphenol. Iodine is greatly reduced in its action by serum, as also are the chlorine compounds. In the case of diamino-acridine (proflavine) and its derivatives and also diamino-methyl-acridinium chloride (acriflavine),¹ it was found that serum increased the bactericidal action on *Staphylococcus aureus*, *B. anthracis*, *Streptococcus*, *B. coli*, *B. typhosus*, *B. pyocyaneus*, timothy grass bacillus, and also on anaerobes. *This is the only group of potent bactericides so far investigated which does not suffer marked reduction in potency in the presence of serum.*

THE TIME RATE OF LETHAL ACTION

The rate of progress of sterilisation with antiseptics of different types has been investigated by making subcultures from the antiseptic mixtures in fluid medium at intervals of two, five, and twenty-four hours. The subcultures were made by inocu-

¹ This compound was prepared by Benda for Ehrlich, and was originally called "trypaflavin" on account of its therapeutic action in experimental trypanosome infections.

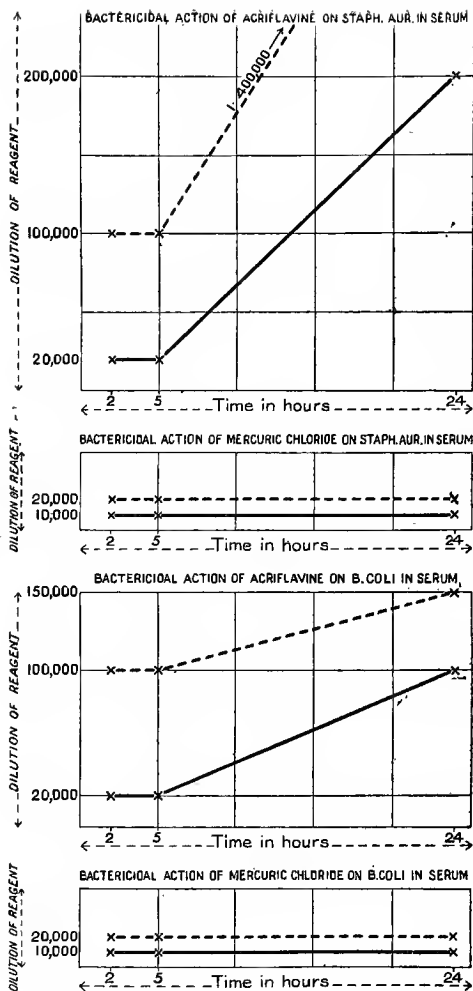
lating a loopful of the mixture into 10 c.cm. of peptone water, and also by stroking agar, and then incubating for forty-eight hours at 37° C. The following table (III) gives the results of representative experiments. Thus, it is clear that, as compared with the other substances tested, the maximum bactericidal action of the acridinium compound, acriflavine, is developed comparatively slowly—diamino-acridine (proflavine) behaves similarly—whereas mercury perchloride, chloramine-T, and phenol, all produce their maximum effect within two hours, and no significant change occurs subsequently. The acridine derivatives have by that time exerted only a restraining effect on the multiplication of organisms in the higher dilutions, which will become sterile later on; this is shown by the relative scantiness of growth when a subculture is made on agar from the tubes containing those particular concentrations. The progressive action of the acridine compounds is evident both in the watery (peptone) medium and also in serum. The graphic representations in Table IV (all to the same scale) of the results in serum demonstrate strikingly the differences between the progress of sterilising action of diamino-methyl-acridinium chloride (acriflavine) on the one hand, and mercury perchloride on the other; in this respect the mercury salt behaves similarly to the other antiseptics tested, although, of course, much higher concentrations of the others are required, as shown in Table III. After two hours' action the difference between the lethal con-

TABLE III
RATE OF PROGRESS OF STERILISATION.

Time in Hours.	Acriflavine.		Chloramine-T.		Mercury perchloride.		Phenol.	
	0.7 per cent. Peptone Water.	Serum.	0.7 per cent. Peptone Water.	Serum.	0.7 per cent. Peptone Water.	Serum.	0.7 per cent. Peptone Water.	Serum.
<i>B. coli.</i>	+	1:1000	1:10,000	1:5000	1:4,000,000	1:20,000	1:500	1:500
	-	?	1:5000	1:1000	1:2,000,000	1:10,000	?	?
	+	1:2000	} Ditto as above	} Ditto as above	} Ditto as above	} Ditto as above	} Ditto as above	} Ditto as above
	-	1:1000						
	+	1:2000						
<i>Staphylococcus aureus.</i>	+	1:1000	1:10,000	1:1000	1:1,000,000	1:20,000	1:250	1:250
	-	?	1:5000	1:500	1:660,000	1:10,000	?	?
	+	1:10,000	1:10,000	1:500	} Ditto as above	} Ditto as above	} 1:500 1:250 1:500	} 1:250 ? 1:500
	-	?	1:5000	?				
	+	1:100,000	1:10,000	1:500				
24 hours	-	1:400,000	1:10,000	1:500	} above	} above	} 1:500 1:250	} 1:500 1:250
	-	1:20,000	1:5000	?				

+ = Growth in subculture in peptone water. -- = No growth. ? Denotes that the lethal concentration was not determined.

TABLE IV



× Indicates actual observations. The heavy line shows lethal concentrations. The broken line shows non-lethal concentrations.

centration of mercury perchloride and of acriflavine is practically negligible ; but subsequently the effect of the acridine compound becomes at least ten to twenty times more potent than that of the mercury salt, and even a further increase in the sterilising action has been observed to occur from the twenty-fourth to the forty-eighth hour (see Table III).

Brilliant green is also slow in action, a feature which has been observed in the case of the allied methyl-violet compounds by various observers.

THE EFFECT OF VARIATIONS IN THE NUMBER OF ORGANISMS

When the effect of a great increase in the number of organisms introduced into the antiseptic solution is tested in the case of the acridine compound (acriflavine), it is found that only a very slight difference is produced in the ultimate result. The following is an example :

ESTIMATION OF BACTERICIDAL CONCENTRATION OF ACRIFLAVINE FOR *B. COLI* IN OX SERUM (57° C.)

The tests were carried out as previously described ; but three parallel series were set up, which differed only in the amount of organisms used for inoculation. Of a twenty-four-hour peptone-water growth of *B. coli* (Escherich) each tube (1 c.cm. volume) received respectively, in Series A, 0.1 c.cm. 1 : 20,000 dilution ; Series B, 0.1 c.cm. 1 : 1,000 dilution ; Series C, 0.1 c.cm. undiluted culture. The mixtures were kept at 37° C., and sub-cultures made on agar at intervals. The controls contained no acriflavine. The results are shown in Table V.

TABLE V

Time of Subculture	Dilution of B. coli Culture used for Inoculation.			Controls.
	A 1: 20,000.	B 1: 1000.	C Undiluted.	
At once .	$\begin{cases} + & 1: 100,000 \\ - & ? \end{cases}$	$\begin{cases} + & 1: 10,000 \\ - & ? \end{cases}$	$\begin{cases} + & 1: 10,000 \\ - & ? \end{cases}$	15 colonies in loopful from Series A; ∞ from C.
2 hours .	$\begin{cases} + & 1: 100,000 \text{ D} \\ - & ? \end{cases}$	$\begin{cases} + & 1: 40,000 \text{ D} \\ - & 1: 20,000 \end{cases}$	$\begin{cases} + & 1: 40,000 \text{ D} \\ - & 1: 10,000 \text{ D} \\ & ? \end{cases}$	No increase as compared with "at once."
5 hours .	$\begin{cases} + & 1: 100,000 \text{ D} \\ - & ? \end{cases}$	$\begin{cases} + & 1: 100,000 \text{ D} \\ - & 1: 40,000 \end{cases}$	$\begin{cases} + & 1: 40,000 \text{ D} \\ - & 1: 20,000 \end{cases}$	Increase.
24 hours .	$\begin{cases} + & 1: 400,000 \text{ D} \\ - & 1: 100,000 \end{cases}$	$\begin{cases} + & 1: 400,000 \text{ D} \\ - & 1: 40,000 \end{cases}$	$\begin{cases} + & 1: 100,000 \text{ D} \\ - & 1: 40,000 \end{cases}$	Increase.

+ = Growth. - = No growth. D = Decrease in number of colonies as compared with control made from same series "at once." ? = Concentration not determined.

Thus, a twenty-thousand-fold increase in the inoculating dose causes only about a two-and-a-half-fold increase in the amount of antiseptic necessary to produce sterilisation. Similar results were obtained with *Staphylococcus aureus*, but with the heavy inoculation distinct proliferation preceded the lethal action.

BACTERIOSTATIC ACTION

Nutrient mixtures, such as peptone water and serum, to which the organic antiseptics have been added, may remain perfectly clear after inoculation, thus showing that no growth of the organisms has occurred and yet many days later a subculture may show that living bacteria are still present. This property of inhibiting proliferation, or "bacteriostatic action" as it has been termed by Gildersleeve

(see Churchman), is a remarkable phenomenon. Under the conditions produced here proliferative activity seems to be "damped down" to a minimum, whereas vegetative life apparently does not suffer marked interference.

It is clear from the above experiments that the conception of potency of bactericidal and antiseptic action must be clearly dissociated from rapidity of sterilisation.

PRACTICAL AND THEORETICAL BEARINGS OF THE INVESTIGATIONS

THE THERAPY OF LOCAL INFECTIONS

It is not intended to enter here into any detailed general consideration of the properties which antiseptic substances must possess in order that they may be effective in the treatment of septic wounds and other localised infective conditions; but the bearings of the observations above described on this important problem are worthy of mention. Many observers, following more or less the classical procedure advocated by Rideal and Walker, estimate the bactericidal value of a compound according to the effect produced by contact of the antiseptic with the organisms for a comparatively brief period—up to fifteen minutes. Accordingly, in the case of antiseptics such as the flavine compounds (acriflavine and proflavine) or brilliant green, a fifteen minutes' test on the Rideal-Walker plan, especially in a watery medium, will reveal so low a "coefficient" as to discourage those who would seek

to estimate the therapeutic possibilities of a substance according to this criterion. The separate consideration of these two functions of rate and potency will play an important part in determining the value of antiseptics for particular applications, when these become more extensively the object of scientific investigation than has been the case in the past.

As Browning, Gulbransen, and Thornton have pointed out, the question as to what constitute the most advantageous properties of an antiseptic, depends almost entirely on the particular purpose for which it is used. For the sterilising of material outside the human body, as in disinfecting garments, instruments, etc., practically the sole necessity is to destroy the organisms, but in the case of therapeutic application in a wound, the properties of the antiseptic must be so adjusted as to ensure efficient action on the bacteria, while at the same time giving rise to a minimum of tissue destruction or interference with those protective and proliferative functions upon which healing depends. A cogent illustration of the point in question is the harmlessness of proflavine and acriflavine when solutions of 1:1000 strength are brought into contact with the peritoneum. It appears most likely that in contaminated wounds, especially when treated early before the symptoms of sepsis have had time to develop—the period at which effective intervention is of most value—actual destruction of the bacteria through the sole agency of the antiseptic is not essential, it being necessary only to

ensure that no significant multiplication or pathogenic action on the part of the implanted organisms shall occur ; in other words, a concentration powerful enough merely to produce inhibition is sufficient. Antiseptics which are non-toxic and relatively harmless to the tissues, such as certain of the organic types, *e.g.* the flavines and brilliant green, are highly suited for this purpose (see Leitch ; Browning, Gulbransen, Kennaway and Thornton ; Ligat ; Browning, Gulbransen, and Thornton ; Drummond and McNee ; Carslaw ; Browning and Ligat), and when, as in the case of the flavine compounds, serum enhances rather than diminishes the bactericidal power, we have the nearest approach to an ideal, continuously acting, therapeutic antiseptic which has so far been realised.

These are, however, but a few of the points which require consideration, since it is only recently that an effort has been made to conduct systematic investigations in the laboratory with a view to determining those properties of antiseptics which may be taken as an index of their suitability for therapeutic use. It must be borne in mind that the vulnerability of different tissues may vary greatly ; in connection with the treatment of infections of delicate mucous membranes, *e.g.* gonorrhoea, it is important that the substance employed should by itself produce a minimum of irritation. While acriflavine and proflavine are in general equal in their antiseptic properties, the latter is tolerated by the rabbit's conjunctiva in a concentration three

times greater than the former ; thus an indication is afforded that proflavine will prove the preferable agent where chances of irritation must be avoided. Again, the influence exerted on connective tissue requires to be studied, and this may be done by means of subcutaneous injection, whereby the substance enters the tissue spaces and conditions are produced materially different from those in which the action of the drug is tested on the tissues exposed in a wound. It is of great interest that when a suitable concentration of brilliant green is injected subcutaneously, marked local accumulation of serous fluid occurs, which disappears spontaneously after persisting for weeks. The general result emerging from the biological investigation of the acridine and triphenylmethane compounds points to these substances as marking a notable advance in the search for bodies which shall possess a high degree of antiseptic power for the common pathogenic bacteria combined with a relatively slight toxicity for living tissues. These characters offer a promising field for therapeutic application which has scarcely yet begun to be explored. An indication as to what may be attained in one direction is afforded by the results of Mackie (unpublished), who, by means of brilliant-green solution (1 : 500–1 : 250), sprayed over the fauces, tonsils, and pharynx thrice daily for a week, succeeded in eliminating diphtheria bacilli from the throats of chronic carriers whose infections had resisted various other forms of local treatment over periods of many months.

THE SELECTIVE ACTION OF ANTISEPTICS

As has been seen, when the bactericidal properties of various antiseptics for several species of bacteria are investigated remarkable differences often appear. Instances of this phenomenon of selective bactericidal action have been long known (Rozsahegyi), but little attention has been devoted to the possibilities thus afforded for studying the influence of chemical and physical factors on such relatively simple examples of vital processes as bacteria present. The practical applications have also been comparatively neglected. Stilling appears to have been the first to note the powerful antiseptic action of ethyl violet for staphylococci, and he suggested the employment of a mixture of allied dyes as therapeutic agents in infective conditions, especially of the eye. Conradi and Drigalski utilised crystal violet in their culture medium partly on account of its selective action, as a means of suppressing the Gram-positive cocci present in faeces, while *B. coli* and *B. typhosus* were enabled to grow freely. Dreyer, Kriegler, and Walker noted the powerful action of the analogous dye, methyl violet, on staphylococci and they drew attention, as likewise did Churchman, using "gentian violet," to the great difference in the susceptibility of Gram-positive organisms such as *Staphylococcus aureus*, which was highly susceptible, whereas Gram-negative bacilli, *e.g.* *B. typhosus* and *B. pyocyaneus*, were resistant (see further Simon and Wood). This rule is, however, by no means

universal, since, as has been shown above, the Gram-negative organism *V. cholerae* is relatively susceptible, while of the Gram-positive types, pneumococci and some strains of streptococci are resistant (Churchman; Krumwiede and Pratt); Churchman and Michael have also pointed out that certain strains of enteritidis type are susceptible. Churchman emphasised the possibilities thus afforded for the isolation of the violet-resistant organisms from mixtures with susceptible types. But, since in most instances the difficulties in separation lie in the opposite direction, *i.e.* the Gram-negative bacilli tend to overgrow the others, this fact is of no great practical importance except for the isolation of tubercle bacilli. Drigalski and Conradi appear to have originally made the highly important observation that *B. coli* of the usual type was more readily inhibited by malachite green and brilliant green than *B. typhosus* was, but Loeffler seems to have been the first to use this property practically in the case of malachite green (see p. 97). No highly advantageous mode of application was devised, however, and the selective character of these substances as discriminators between *B. coli* and *B. typhosus* became more or less discredited, until the use of brilliant green in fluid medium was adopted by Browning, Gilmour, and Mackie (see p. 113).

“LAWS” OF ANTISEPTIC ACTION

The greater part of the important work which has hitherto been done on the bactericidal effect of

chemical and physical agencies has been directed toward the mathematical representation of the velocity of the process ; it has been found in general that disinfection proceeds according to the law that the number of organisms destroyed in unit time is a constant proportion of the number alive at the beginning of the period of investigation. According to the conclusion reached by Chick one must assume that all the organisms in a culture possess similar resistance to the bactericidal agent. A particular organism may at a certain moment be in a phase of resistance which is above or below the average, but even if its resistance be temporarily elevated it soon falls to the average again. Individuals permanently above and below the average are held not to exist ; if this be true, then bacteria differ notably from most living objects and possibly their relatively simple organisation may afford the explanation, although it must be remembered that no proof has been afforded that the protein which constitutes bacteria is less complex in its constitution than that of higher forms. The comparative slowness with which lethal action occurs in the case of certain of the antiseptics here investigated, *e.g.* the acridine compounds and triphenyl-methane derivatives, should afford exceedingly favourable conditions for the further investigation of this problem. It is particularly interesting in this connection that when the action of diamino-methyl-acridinium chloride on staphylococcus suspended in serum was tested, employing large doses for inoculation (0.1 c.cm. of

a twenty-four-hour peptone water culture to 1 c.cm. of serum containing the antiseptic) definite proliferation was observed to occur prior to death of the organisms when the mixture was incubated at 37° C.

ACQUIRED RESISTANCE TO ANTISEPTICS

The failure to produce markedly elevated resistance to acridine compounds and brilliant green by prolonged growth and repeated subculture in media containing these reagents for a period of over six months, also suggests that the susceptibility of given strains is a remarkably constant property. From the therapeutic point of view this is a highly satisfactory observation, although naturally resistant strains exist (*e.g.* certain coliform bacilli toward flavine bodies—Drummond and McNee). In the case of other organisms and different antiseptics it has been possible to develop resistant strains *in vitro* (Shiga, Marks), and Morgenroth and Kaufmann have produced resistant pneumococci *in vivo* by means of ethyl-hydrocuprein in analogy to the work of Ehrlich and Browning and others in trypanosome infections. The high degree of selective action of many of these substances on one organism as compared with another points, in these cases, to the existence of some highly specialised relationship between the antiseptic and the constituents of the organisms; in this connection the disparity of the lethal properties of hexamethyl violet for staphylococcus and for *B. coli*, on the one hand, and, on the other, the

equality of action of mercuric chloride on these organisms indicates a radical difference between the points of attack of the two bactericidal agents. The parallel between resistance to brilliant green and the capacity to ferment inosite on the part of certain members of the coli group (p. 211) is a remarkable biological correlation, and the phenomenon of bacteriostatic action (see p. 83) raises an interesting problem as to the duration of life of a non-sporing bacterium.

LIST OF PRACTICAL APPLICATIONS OF SELECTIVE ANTISEPTICS FOR THE ISOLATION OF PARTICULAR PATHOGENIC ORGANISMS AND THE SUPPRESSION OF CONTAMINATIONS

Isolation of typhoid - paratyphoid bacilli from mixtures with coliform bacilli	}	Brilliant Green (p. 95).
Ditto, when coliform bacilli of type resistant to brilliant green by itself are present		
Isolation of <i>B. diphtheriae</i> and diphtheroid organisms, especially where abundant staphylococci are present or profusely growing Gram-negative bacilli	}	Brilliant Green <i>plus</i> Telluric Acid (p. 108).
Isolation of <i>B. mallei</i>		
Isolation of staphylococci from mixtures with coliform bacilli	}	Telluric Acid (p. 141).
Suppression of <i>B. pyocyaneus</i> in order to permit isolation of streptococci and staphylococci		
Suppression of "spreader" types of <i>B. proteus</i> (<i>urobacillus septicus</i>) in order to permit isolation of streptococci, etc.	}	Thorium Nitrate (p. 147).
	}	Silver Nitrate (p. 71).
	}	Telluric Acid (pp. 143, 149), or Thallium Acetate (p. 147).
	}	Thallium Acetate (p. 147).

When solid media containing differential anti-septics are employed for isolating bacteria, it is important to make several successive subcultures on the differential medium, on each occasion inoculating from a single colony. The reason for this is that the contaminating bacteria tend to persist in a latent state in the early culture, and then grow when the organisms are transferred to a medium suitable for their proliferation. This, of course, applies even apart from the use of differential media, *e.g.* in the separation of *B. diphtheriae* from streptococci.

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VI

THE ISOLATION OF TYPHOID-PARATYPHOID BACILLI FROM FAECES, ETC., BY MEANS OF ENRICHMENT WITH BRILLIANT GREEN AND TELLURIC ACID IN FLUID MEDIUM ; THE EXPERIMENTAL BASIS OF THE METHOD AND A REVIEW OF RESULTS

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Papers incorporated

Browning, Gilmour, and Mackie, *Journal of Pathology and Bacteriology*, vol. xviii. 1913, p. 146; and *Journal of Hygiene*, vol. xiii. 1913, p. 335.

Browning, Mackie, and Smith, *Journal of Pathology and Bacteriology*, vol. xix. 1914, p. 127.

Browning and Thornton, *British Medical Journal*, August 14th, 1915, and May 13th, 1916.

THE evidence recently accumulated as to the effect of anti-typhoid inoculation and measures based on the detection of "carriers" has definitely strengthened the view that organisms of the typhoid-paratyphoid group are important pathogenic agents. Thus, methods for discovering these organisms con-

tinuc to command wide attention. In the case of blood and urine, cultivation is a relatively simple matter, as the bacilli are likely to be present in pure state. On the other hand, there is in faeces an exceedingly heterogeneous mixture of different bacterial types along with dead organic matter; nevertheless it is frequently highly important to be able to determine the presence of typhoid or paratyphoid bacilli in the intestinal contents, since a patient may come under observation when cultures both from the blood and urine are negative, also the diagnostic significance of the Widal reaction may be complicated by anti-typhoid and paratyphoid inoculation. The number of procedures which have been advocated for recovering these organisms from faeces indicates the limited value of the majority of them. Difficulty arises wherever specific bacteria are scanty in comparison with the other viable organisms present. The old method of spreading the faeces over an extensive area of solid medium in Petri capsules, so as to obtain a large number of isolated colonies, left much to be desired, even when the medium was so constituted that cocci were inhibited and the common varieties of *B. coli* could be distinguished by a colour reaction due to the presence of a fermentable sugar and an indicator, as in Drigalski and Conradi's, Endo's, and MacConkey's media. None of these media interferes notably with the growth of coliform bacilli, hence where the typhoid or paratyphoid organisms are small in number a very large surface must be spread in order that one

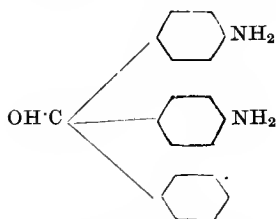
colony of the pathogenic bacillus may be present among the isolated colonies which develop. A further difficulty consists in the fact that numerous coliform bacilli produce pale growths on these media and consequently yield "likely" colonies whose fermentation reactions require to be tested. Thus Harvey in his important work on the detection of carriers in the Indian army used three 9-inch plates of Conradi's medium for each sample of faeces and every likely colony was picked off and investigated. A distinct advance was effected by the discovery of antiseptic agents which acted more powerfully on *B. coli* than on the typhoid bacillus. Drigalski and Conradi appear originally to have published observations regarding the effect of such differential antiseptics on organisms of the typhoid-coli group, and they mention both malachite green and brilliant green as exerting a more marked inhibitory action on *B. coli* than on *B. typhosus*. Loeffler, however, seems to have been the first to make practical use of this property, and he employed malachite green in agar; Savage and also Lentz and Tietz made extensive use of this reagent. Conradi later recommended a mixture of brilliant green and picric acid, and Fawcett added bile salts; but these media do not appear to have come into general use. The fact that brilliant green and malachite green are more powerfully bactericidal for *B. coli* than for *B. typhosus* had not been universally accepted (Kathe and Blasius); Browning and Gilmour, however, confirmed Drigalski and Conradi's main

observation in the course of systematic investigations on the antiseptic action of basic benzol-derivatives.

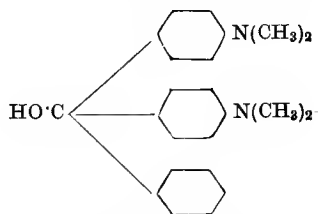
THE DIFFERENTIAL ANTISEPTIC ACTION OF DIAMINO-TRIPHENYL-METHANE DYES ON ORGANISMS OF THE TYPHOID-COLI GROUP

A certain amount of confusion concerning the action of these dyes has been created by bacteriological workers who have not regarded the chemical constitution of the substances which they employed; malachite green is the tetramethyl derivative of diamino-triphenyl-methane-carbinol and is commonly employed as the oxalate, or the double chloride with zinc; brilliant green is the sulphate of the tetraethyl derivative; the unsubstituted diamino-triphenyl-methane compound (hydrochloric acid salt) is known as Doebner's violet.¹

CONSTITUTION OF DIAMINO-TRIPHENYL-METHANE DERIVATIVES

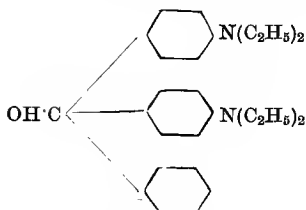


Unsubstituted compound—
Doebner's Violet (carbinol)

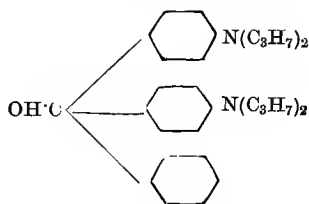


Tetramethyl compound—
Malachite Green (carbinol)

¹ Bittermandelölgrün, China green, solidgrün, are other names for malachite green; smaragdgrün krist. is brilliant green; anilin green (aldehyde green) is a different chemical compound.



Tetraethyl compound—
Brilliant Green (carbinol)



Tetrapropyl compound—
Propyl Green (carbinol)

A further compound, "propyl green," a specimen of which was prepared by the action of propyl iodide on benzaldehyde (unpublished work of E. L. Kennaway) has also been tested. The influence of chemical constitution on bactericidal power is brought into especial prominence by comparing the action of these bodies on *B. typhosus*, *B. coli*, and *Staphylococcus aureus*, as shown in Table I. Thus it appears clearly that brilliant green is the most potent of all these substances in its action on *B. coli*, and, further, *B. coli* is more susceptible to this dye than is *B. typhosus*; brilliant green is in general the most highly differential of these substances in its action on the two organisms. Propyl green, while about twice as bactericidal as Doebner's violet toward *B. coli* and *B. typhosus*, is almost a hundred times as active as the latter toward *Staphylococcus aureus*. This marked difference in effect on the two groups of organisms shown by propyl green is similar to that exhibited by the triphenyl-methane compound hexamethyl (crystal) violet (Churchman, see also page 69). Experiments (unpub-

TABLE I

THE BACTERICIDAL ACTION OF DIAMINO-TRIPHENYL-METHANE DYES ON *B. TYPHOSUS*, *B. COLI*, AND *STAPHYLOCOCCUS AUREUS*

Amount of 1 : 100 solution of the dye in c.cm. added to 1 c.cm. of 0·7 per cent. peptone water neutral to litmus, and inoculated with 0·1 c.cm. of a 1 : 20,000 dilution of a twenty-four-hour peptone-water culture (37° C.).

+ = growth obtained } in subculture made after 24 hours at
- = sterile } 37° C.

Dye.	<i>B. typhosus</i> .	<i>B. coli</i> <i>communis</i> (<i>Escherich</i>).	<i>Staphylo-</i> <i>coccus aureus</i> .
Doebner's Violet	0·05+	0·05 +	0·0025 +
(Hydrochloride)	0·1 —	0·1 —	0·005 —
Malachite Green	0·005 +	0·0035 +	0·000005 +
(Oxalate)	0·0075 —	0·005 —	0·00001 —
Brilliant Green	0·0035 +	0·0005 +	0·000005 +
(Sulphate)	0·005 —	0·00075 —	0·00001 —
Propyl Green	0·02 +	0·02 +	0·00003 +
(Hydrochloride)	0·04 —	0·04 —	0·00004 —

Note.—The experimental results are subject to considerable variations, but the above represents the average.

lished) by R. Gulbransen on different salts of the brilliant-green base, viz. the hydrochloride, mucate, oxalate, tartrate,¹ have shown that all these substances are practically equal to the sulphate in their antiseptic action, thus the acid radical appears to exert no marked determining influence in this respect.

Tidy and Dunn have established the superiority of brilliant green over malachite green as regards

¹ We are indebted to the Imperial College of Science and Technology for the preparation of these compounds.

selective action by another method: they made mixtures of cultures of *B. typhosus* and *B. coli* in varying proportions, and found that in the brilliant-green series inoculated with coli-typhoid mixture in the ratio of 100:1 they were able to isolate *B. typhosus* subsequently, whereas with malachite green they failed when the mixture contained more than 10 coli organisms to 1 typhoid bacillus.

The above results were obtained with a typical strain of *B. coli communis* (Escherich); the term *B. coli*, however, includes a wide variety of organisms which differ markedly in their biological characters—fermentation of sugars, etc.—a point the clear understanding of which is due mainly to the work of MacConkey. Accordingly, Browning, Gilmour, and Mackie tested the action of brilliant green on fifty-four different strains of *B. coli* of types commonly found in faeces, which had been isolated from human faeces, urine, appendix abscesses, etc.,¹ and also on thirteen strains of *B. proteus* all of human origin, and compared them with twenty-one strains of *B. typhosus*, as well as with paratyphoid and dysentery organisms.

The results were as follows:

Organisms less resistant to brilliant green than B. typhosus:

B. No. 71, *B. coli communis*, *B. neapolitanus*, *B. Schafferi*, *B. vesiculosus*, *B. Grünthal*, *B. coscoroba*, *B. 106*, *B. coli anaerogenes* (six strains), some paracolon bacilli (non-lactose fermenters,

¹ The classification of coliform bacilli is that adopted by MacConkey.

which do not form indol and which differ from the paratyphoid group either in being non-motile or in not fermenting dulcitol), *dysentery bacilli* (confirmed by Tidy and Dunn, Douglas and Colebrook), and *B. faecalis alkaliganes*, also B. 74.

Organisms of slightly less resistance or practically equal to B. typhosus :

B. proteus (thirteen strains).

Organisms more resistant than B. typhosus :

Paratyphoid group, Gaertner's bacillus, and certain types of *B. coli* (inositol-fermenters, *e.g.* *B. lactis aerogenes* and B. No. 67), also some non-indol-forming types of *B. coli*—certain of the latter fermenting lactose only after mutation. Certain paracolonic bacilli, including the pseudo-paratyphoid B (see p. 24). *B. pyocyaneus* types.

Torrey independently observed the marked resistance of the paratyphoid-enteritidis group; Krumwiede and Pratt have also confirmed the resistance of the typhoid-paratyphoid group to brilliant green and the super-resistance of organisms such as *B. lactis aerogenes*. The statement which has been made again lately (Robinson), that concentrations of brilliant green sufficient to interfere with the growth of *B. coli* also inhibit *B. typhosus* in practically equal degree, must rest on some peculiarity in the experimental conditions. The resistance of different strains of *B. typhosus* toward brilliant green varies somewhat, of course, and specially susceptible cultures do occur. The author has observed one such strain ("Fisher," see Table II), which upon repeated examinations over a long

TABLE II

SHOWING THE ACTION OF BRILLIANT GREEN ON DIFFERENT STRAINS OF *B. TYPHOSUS*

Plates of 10 c.cm. peptone-water agar (neutral to litmus) *plus* varying amounts of brilliant-green solution, inoculated with one stroke of a faintly opalescent emulsion of agar growth (twenty-four hours at 37° C.) of each of the cultures.

Amount of Brilliant Green 1: 10,000 per 10 c.cm. medium.		Resulting growths after 48 hours at 37° C.					
		Strains of <i>B. typhosus</i> .					
		Stock.	Fisher.	No. 500.	No. 521.	No. 551.	<i>B. coli</i> <i>communis</i> (Fischerich).
0·3 c.cm.	.	abundant	abundant slightly inhibited	abundant	abundant	abundant	abundant 3 colonies
0·5 c.cm.	.	"	markedly inhibited	"	"	"	6 colonies
0·8 c.cm.	.	"	no growth	markedly inhibited	distinctly inhibited	slightly inhibited	1 colony
1·2 c.cm.	.	"	1 colony	3 colonies	20 colonies	50 colonies	no growth
1·6 c.cm.	.	slightly inhibited					
Control: no brilliant green .		abundant	abundant	abundant	abundant	abundant	abundant

period (years) has always manifested marked susceptibility toward the dye; but such highly sensitive races of *B. typhosus* appear to be uncommon.

THE EFFECT OF REACTION OF THE MEDIUM ON THE DIFFERENTIAL ANTISEPTIC ACTION OF BRILLIANT GREEN

It is well known that the majority of diamino-triphenyl-methane dyes, including brilliant green, are exceedingly sensitive to alkali, being converted into the leucobase and decolorised. From the bacteriological standpoint, in addition, the behaviour of the organisms under different reactions of the medium had to be considered; accordingly, the effect of variations in reaction were tested both in peptone-water medium and in agar. The following is an example:

Two per cent. peptone-water agar was rendered neutral to phenol-phthalein with caustic soda and then varying amounts of phosphoric acid were added. After the adjustment of the reaction brilliant green was added and plates were poured. Stroke inoculations were made on the surface of the medium with one loopful of faintly opalescent suspensions of twenty-four-hour cultures in 0.85 per cent. NaCl solution. Table III shows the resulting growth on the various plates after incubation for forty-eight hours at 37° C.

The results show that a neutral or very slightly alkaline reaction to litmus (much less than produces an alkaline reaction to phenol-phthalein) is the optimum for eliciting the differential antiseptic

TABLE III
EFFECT OF REACTION OF MEDIUM

Amounts of Brilliant Green 1 : 10,000 per 5 c.cm. agar.	Neutral to phenol-phthalein.		Midway between the neutral point to phenol- phthalein and litmus.		Neutral to litmus.		2.8 per cent. (normal) acid to phenol-phthalein.	
	Growth of		Growth of		Growth of		Growth of	
	B. coli.	B. typhosus.	B. coli.	B. typhosus.	R. coli.	B. typhosus.	E. coli.	B. typhosus.
0.12 c.cm.	abundant	abundant	very scanty	abundant	slight inhibi- tion	abundant	abundant	abundant
0.2 c.cm.	scanty	"	"	"	very scanty	"	"	"
0.3 c.cm.	none	scanty	"	very scanty	very scanty	"	"	none
Control: no bril- liant green	abundant	abundant	abundant	abundant	abundant	abundant	abundant	abundant

* Conradi's brilliant-green picric-acid medium is 3 per cent. acid. Phosphoric acid was employed because Conradi recom-
mended it for the preparation of his medium; but hydrochloric acid gave similar results.

effect; on the other hand acid, short of an amount which by itself prevents the growth of both organisms, increases the growth of *B. coli* and abolishes the differential action in favour of *B. typhosus*. Accordingly, our medium has throughout been adjusted so as to react neutral or very faintly alkaline to litmus paper. Robinson and Rettger found that 2 per cent. peptone water, without correction of the reaction, decolorised the brilliant green in twenty-four hours, accordingly they recommend that the reaction be adjusted to + 1. We have not found any decolorising effect with the medium described below; but differences doubtless depend on the brand of peptone employed; accordingly, *workers should determine for themselves the reaction which gives the best differential antiseptic effect* with cultures of *B. coli* and *B. typhosus*. The antiseptic action of the dye varies in different batches of agar; this may be due in part to the reaction, for the titration of agar medium is scarcely an operation of high precision. Krumwiede, Pratt, and McWilliams point out also that the sediment of cloudy agar diminishes the antiseptic effect.

NUMERICAL EXPERIMENTS ON THE ISOLATION OF *B. TYPHOSUS* FROM ARTIFICIAL MIXTURES WITH FAECES BY MEANS OF BRILLIANT GREEN

Such tests have a certain value as indicating the delicacy of the selective antiseptic, since a fixed number of typhoid bacilli can be introduced. The

following experiment was performed by Browning, Gilmour, and Mackie :

A homogeneous emulsion of fresh normal faeces was prepared by rubbing up with two volumes of 0·85 per cent. NaCl solution and straining through wire gauze. A loopful (diameter of the loop 0·4 cm.) of the emulsion was added to a series of 5 c.cm. peptone-water tubes containing varying amounts of brilliant green (1 : 10,000), viz. 0·14, 0·19, 0·24, 0·3 c.cm. ; at the same time a measured amount of a dilution of the twenty-four-hour bouillon culture of a strain of *B. typhosus* of average resistance to brilliant green was introduced. A similar amount of the dilution of typhoid culture when plated on agar yielded two colonies. The number of viable organisms present in the amount of faeces employed in each tube was 2800, as ascertained by plating on agar. Subcultures made from the brilliant-green tubes after incubation at 37° C. for twenty-four hours gave the following growths :

Tube containing	Resulting growth.
0·14 c.cm. brilliant green.	Numerous red colonies (? mixture of <i>B. typhosus</i> with <i>B. coli</i>).
0·19 c.cm. ,, ,,	<i>B. typhosus</i> and red colonies in equal numbers.
0·24 c.cm. ,, ,,	Ditto.
0·3 c.cm. ,, ,,	Abundant colonies of <i>B. typhosus</i> and two red colonies.

Thus, a practically pure culture of *B. typhosus* was obtained from a mixture of about two typhoid bacilli along with 2800 other viable organisms. A control series of brilliant-green cultures of the faeces, without the addition of *B. typhosus*, yielded nothing resembling typhoid bacilli. A direct inoculation of the contaminated faeces on MacConkey's medium gave only a growth of *B. coli*. In a series of similar experiments in which a larger number of typhoid

bacilli (ten) were added, we failed also to recover a typhoid growth by plating directly on MacConkey's medium, although practically pure growths of *B. typhosus* were obtained by means of brilliant green. Robertson and Rettger quote a similar experiment in which *B. typhosus* added in the proportion of 70 to 135,000 viable faecal bacteria was successfully isolated by means of brilliant green. It is scarcely necessary to emphasise the fact that only an *enrichment* method could have effected readily the isolation of *B. typhosus* from such abundant contaminations.

THE ACTION OF TELLURIC ACID ALONE AND IN MIXTURES WITH BRILLIANT GREEN ON THE TYPHOID-COLI GROUP

As has been seen, certain varieties of coliform bacilli are much more resistant than *B. typhosus* to brilliant green; the resistance is especially marked in the case of that group of organisms which ferments inosite, *e.g.* *B. lactis aerogenes*. This property is independent of the other biological characters of the group, *e.g.* indol-formation, fermentation of dulcitol and inulin, Proskauer reaction, etc. When such resistant types are present in the faeces there is the danger that under the influence of brilliant green they will outgrow the typhoid organisms in a fluid medium, although by the use of a *series* of amounts of brilliant green an optimum may be found which enables *B. typhosus* to flourish, while with larger

TABLE IV
CASE M'E. (FÆCES)

10 c.cm. peptone-water medium containing—		Resulting growth in subcultures made after twenty-four hours' incubation at 37° C.
Telluric Acid 1:1000	+ Brilliant Green 1:10,000.	
0 . . .	0.1 c.cm.	Inosite-fermenters + pale colonies of a coliform B.
	0.2 „	Inosite-fermenters + pale colonies of a coliform B. + B. typhosus.
	0.35 „	Inosite-fermenters + pale colonies of a coliform B. + B. typhosus (inosite-fermenters more abundant).
	0.5 „	Inosite-fermenters + coliform B.
	0.7 „	Pure culture of inosite-fermenter.
0.33 c.cm.	0.1 „	No growth.
	0.2 „	Pure B. typhosus.
	0.35 „	„ „
	0.5 „	„ „
	0.7 „	Almost pure growth of B. typhosus.

B. typhosus present in direct plate culture.

amounts of the dye the inosite-fermenter overgrows everything (see Table IV—a case examined by Browning, Mackie, and Smith). Browning, Mackie, and Smith, when testing the antiseptic action of telluric acid¹ on organisms of the typhoid-coli group, obtained the striking result that the inosite-fermenters were the most susceptible of all to this reagent. B. typhosus is in general relatively resis-

¹ Potassium tellurate was employed at first, but was found to alter rapidly; telluric acid is stable and keeps indefinitely in the form of a 1 per cent. watery solution, sterilised by heat, which reacts neutral to litmus.

tant to telluric acid, and the common types of coliform bacilli are intermediate as is shown in Table V.

TABLE V

Stroke cultures were made by inoculating plates of agar medium containing varying amounts of telluric acid with a loopful of thin emulsion of agar cultures (24 hours at 37° C.) of each of the organisms.

10c.cm. peptone-water agar faintly alkaline to litmus plus the following amounts of telluric acid 1 per cent.	Resulting growths (48 hours at 37° C.) after inoculation with		
	B. typhosus.	B. coli communis (Escherich).	Inosite-fermenter (No. 67 MacConkey).
0.05 c.cm.	abundant growth	abundant growth	very marked inhibition
0.08 „	„	„	no growth
0.13 „	„	slight inhibition	no growth
0.2 „	3 colonies	no growth	no growth
Control without telluric acid	abundant growth	abundant growth	abundant growth

The behaviour of mixed cultures of B. typhosus, B. coli, and inosite-fermenter when inoculated into fluid medium containing (a) brilliant green, (b) brilliant green plus telluric acid, (c) telluric acid alone, is well shown in the following experiment (Browning, Mackie, and Smith).

Fairly dense suspensions, of approximately equal opacity, of pure twenty-four-hour agar cultures of *B. typhosus*, *B. coli*, and inosite-fermenter were prepared and were mixed in the following amounts in Experiment I: *B. typhosus* 1 volume, *B. coli* 50 volumes, inosite-fermenter 50 volumes; in Experiment II the proportions were, *B. typhosus* 1 volume, and of the other two

organisms each 10 volumes. Two series of 10 c.cm. tubes of 2 per cent. peptone water (neutral to litmus) containing respectively (a) varying amounts of brilliant green, (b) varying amounts of brilliant green along with telluric acid, were inoculated with one loopful of the mixed suspensions and were then incubated for twenty-four hours at 37° C. At the end of that time subcultures were made on plates of MacConkey's agar medium.

The results are shown in Table VI.

TABLE VI

10 c.cm. 2 per cent. peptone-water medium (neutral to litmus) containing—		Inoculated with 1 loopful of mixed suspension of pure cultures in the following proportions and kept twenty-four hours at 37° C.:			
Telluric Acid 1: 1000	+ Brilliant Green 1: 10,000.	I		II	
		B. coli communis	. 1 .	. 1	
		Inosite-fermenter	. 1 .	. 1	
		B. typhosus	. $\frac{1}{10}$.	. $\frac{1}{10}$	
		Resulting growth of subculture on MacConkey's medium.			
		I	II		
0 . .	{	0.17 c.cm.	Mainly inosite-fermenter — some colonies of B. coli.	Pure growth of inosite-fermenter.	
		0.3 „	Pure growth of inosite-fermenter.	„	
		0.42 „	„	„	
		0.6 „	„	„	
0.3 c.cm. .	{	0.17 „	Mainly B. typhosus — scanty colonies of B. coli.	Almost pure growth of B. typhosus.	
		0.3 „	Colonies of B. typhosus and inosite-fermenter in equal numbers.	Colonies of B. typhosus and inosite-fermenter in equal numbers.	
		0.42 „	Mainly inosite-fermenter—scanty B. typhosus.	B. typhosus and inosite-fermenter colonies — proportion 1: 3	
		0.6 „	Pure growth of inosite-fermenter.	B. typhosus and inosite-fermenter colonies — proportion 2: 1.	
0.3 „ .	0	Pure B. coli.	Pure B. coli.		

This demonstrates that, in the presence of considerable numbers of inosite-fermenters, telluric acid along with brilliant green is required in order that *B. typhosus* may constitute so significant a proportion of the resulting culture as to enable it to be isolated subsequently. A further point of importance is that the action of telluric acid along with brilliant green cannot be represented as a mere summation of effects; since, as shown by Experiment I, in the presence of telluric acid there was a definite optimum amount of brilliant green necessary for the growth of *B. typhosus*. With larger amounts of brilliant green the growth of the inosite-fermenter preponderated even in spite of the presence of telluric acid. This affords a further striking example of the fact that the growth of a given organism in a mixed culture may be dependent on a very delicate adjustment of conditions (see also Table IV).¹

It has been found by J. F. Smith (unpublished) that *B. paratyphosus* B is more resistant to the mixture of brilliant green and telluric acid than either *B. typhosus* or *B. paratyphosus* A.²

¹ An interesting example of the behaviour of mixed cultures is the mutual inhibition of *B. dysenteriae* (Flexner) and *V. cholerae* when grown together in peptone water (Logie, *Journ. of Hyg.*, vol. xiii. 1913, p. 162).

² Smith has also found that on solid media containing telluric acid along with brilliant green *B. paratyphosus* B in the great majority of cases showed colonies darkened by reduced tellurium, whereas *B. typhosus* and *paratyphosus* A colonies remained pale.

PRACTICAL USE OF BRILLIANT GREEN AND TELLURIC ACID IN FLUID MEDIUM IN CASES OF TYPHOID OR PARATYPHOID INFECTION ¹

In order to utilise the differential antiseptic properties of brilliant green and telluric acid for the isolation of typhoid and paratyphoid bacilli from faeces, Browning, Gilmour, Mackie, and Smith suggested, in addition to the use of direct plating on a medium such as MacConkey's or Endo's, a simple peptone-water medium containing the reagents; for each specimen of faeces they employed (a) a series of tubes containing varying amounts of brilliant green and (b) a similar series to which telluric acid was added also. The following considerations prompted these recommendations: (1) a fluid medium is advisable, because it is only by subjecting the faeces to the antiseptic in fluid that uniform action can be secured. In the case of a solid medium, if the specific organisms are scanty they will occur only where the plate is richly inoculated; but, as is generally recognised, rich inoculation tends to annul the antiseptic effect on the contaminating coliform organisms and, in consequence, the latter are likely to grow exactly where their presence will obscure the typhoid bacilli. On the other hand, with fluid medium there is the danger that B.

¹ Since dysentery bacilli both of Flexner and of Shiga type are less resistant to brilliant green than is the ordinary type of *B. coli*, it follows that the method is not of value for the isolation of the dysentery group. This has been confirmed by Douglas and Colebrook.

typhosus will be overwhelmed if organisms are present which proliferate more vigorously or which produce substances inimical to its growth. Thus, in order to suppress those members of the coli group which are occasionally prominent constituents of faeces and which are more resistant to brilliant green than *B. typhosus*, telluric acid is added to the medium. (2) It is, of course, to be noted that the margin of safety with the differential antiseptics is not very wide; that is, the highest concentration which will permit the growth of *B. typhosus* is not a large multiple of the dose which inhibits *B. coli*, hence the necessity for caution in regard to the adjustment of the quantities of the antiseptics used and the advantage of a series of doses. Thus, the practical problem is how to arrange conditions so as to secure a preponderance of the specific organisms. Now the growth of the typhoid-paratyphoid bacilli depends on factors such as their numbers, the number and type of accompanying organisms, and the amount of organic matter present, none of which can be conveniently standardised beforehand. Since experiments have shown that it is impossible to determine a fixed amount of antiseptic which will lead to the desired result in all cases, a *series* of doses of brilliant green was recommended. The employment of such a series is in our opinion an important feature of the method. Where telluric acid and brilliant green are employed in combination, the effect, as has been seen, is not a mere summation of the actions of the two anti-

septics; hence here also the use of a series of doses is advisable.

Cultures should be made from the faeces as soon as possible after evacuation, preferably within several hours; since when the faeces are allowed to stand for some time the isolation of typhoid bacilli becomes increasingly difficult.¹ When a delay must occur before the faeces are examined, Teague and Clurman's method of preserving typhoid stools for delayed examination by means of emulsifying one part of faeces in two parts of a 30 per cent. dilution of glycerine in 0·6 per cent. NaCl solution, should prove a most valuable procedure; the experience of Benians confirms its reliability (see p. 138). Where the faeces are of normal consistency—for example, in suspected carriers—it is advisable to administer purgatives (calomel, or elaterin) and then examine the resulting fluid evacuations; it is generally agreed that the chances of successful isolation of the specific organisms are considerably increased thereby (see Stokes and Clarke, Tonney, Caldwell and Griffin).

Plates of solid medium containing an indicator, such as Endo's or MacConkey's, are inoculated in the usual fashion, so as to secure a large number of isolated colonies. At the same time the antiseptic enrichment fluid medium is inoculated. The fluid medium consists of 2 per cent. peptone² and 0·5 per

¹ Some workers prefer freshly taken rectal swabs.

² Several different brands of peptone have been used with satisfactory results; Savory & Moore's has been found to serve well.

cent. sodium chloride in distilled water, steamed for three-quarters of an hour and then filtered through ordinary filter-paper.

The reaction should be adjusted, if necessary, till it is only very faintly alkaline to litmus paper—the optimum reaction may depend on the peptone employed (see p. 106); the medium is then distributed in amounts of 10 c.cm. in test tubes and sterilised by steaming or in the autoclave.¹ A stock 1 per cent. solution of brilliant green (sulphate, zinc free) in distilled water is prepared; this keeps for months. Immediately before use, a 1 in 10,000 dilution is freshly made up by adding 0·1 c.cm. of the stock solution to 9·9 c.cm. of distilled water, and then this dilution is added to the peptone water in the following amounts: 0·1, 0·2, 0·35, 0·5, 0·7 c.cm. (corresponding to concentrations of 1 : 1,000,000, 1 : 500,000, 1 : 350,000, 1 : 200,000, 1 : 143,000). In the second series each tube contains, in addition to the brilliant green, 0·4 c.cm. of 1 in 1,000 telluric acid. Where a large number of examinations are carried out at one time the mixture of peptone water and antiseptics may be made up in bulk and then distributed in sterile plugged test tubes (10 c.cm. need not then be accurately measured into each tube).

When for any reason the full procedure cannot be adopted, *e.g.* owing to the necessity for dealing with

¹ Cole and Onslow suggest that their medium prepared from casein digested with trypsin is likely to prove especially suitable for eliciting the differential antiseptic effect of brilliant green.

large numbers of specimens, a simplification consists in using a single concentration of 1 in 200,000 or 1 in 250,000 brilliant green in 10 c.cm. of peptone water. According to the experience of Stokes and Clarke, Smith, Leitch, and Tidy and Dunn this is on the average the optimum concentration for the isolation of the specific organisms.

The amount of faeces to be used.—In regard to this important point, we originally employed a single loopful in each tube; in the case of very fluid faeces a large loop—up to $\frac{1}{8}$ -in. diameter—being employed; solid faeces were emulsified by rubbing up with several volumes of sterile water. The cultures were then incubated for twenty to twenty-four hours at 37° C. and subcultures made on MacConkey's or Endo's medium. Three successive strokes, without recharging the needle, are made from each tube—in this way one 4-in. plate will accommodate the subcultures made from three peptone-water tubes; the plates are then incubated at 37° C. for from eighteen to twenty-four hours, and the colonies examined, as in the case of direct plates.

Leitch advises that a portion of faeces of the size of a pea should be emulsified in 5 c.cm. of bouillon; this emulsion is kept at 37° C. for a few hours, and then two loopfuls of the supernatant fluid are used to inoculate the antiseptic medium; he states that control experiments have shown that a greater number of successful results is obtained in this way than after inoculating the antiseptic medium

directly with the emulsion of faeces. Stokes and Clarke prefer the following procedure: the faeces are emulsified with several volumes of sterile water; the mixture is then allowed to sediment for half an hour, and then five or six large loopfuls of the supernatant fluid are added to the tube of fluid medium. When such a rich inoculation is employed, subcultures are best made after nine hours' incubation at 37° C.

The question of the quantity of faeces employed is an exceedingly important one; with solid medium it is obvious that too much material has been used when isolated colonies in considerable number fail to be obtained. But in the case of fluid medium this criterion cannot be applied; here it is necessary to watch for the occurrence of turbidity and discoloration of the green after incubation; discoloration is, in general, the result of abundant growth of coliform bacilli, and from the tubes in which it occurs the specific organisms are unlikely to be obtained on subculture (Browning and Thornton). The value of a series of varying doses of the dye lies in the fact that when the lower ones show discoloration the next higher doses are then the most likely to yield positive results. Should all the tubes appear clear after twenty-four hours and fail to yield growths in subculture, a positive result may yet be obtained after the fluid cultures have been incubated for a further period. The series of doses is devised so as both to meet the individual features of the particular specimen of faeces and also to permit of considerable

latitude in the amounts used for inoculation by different workers. Torrey has conclusively demonstrated the importance of not using an excessive quantity of faeces; he employed brilliant green in glucose bouillon for the isolation of paratyphoid-enteritidis organisms, and, while using a fixed concentration of the dye, he added varying amounts of faeces to a series of tubes; subcultures were then made after twenty-four hours' incubation at 37° C. He found that "if the paratyphoid or enteritidis bacilli are fairly numerous in the faeces, pure or nearly pure cultures will develop in all of these tubes. If, however, they are present in very small numbers, the specific growth occurs rather more often in the lightly than in the heavily seeded tubes." Inattention to this quantitative factor appears to afford a complete explanation of the poor results obtained by Krumwiede, Pratt, and McWilliams when using brilliant green in fluid medium (see p. 122).

An important feature of the enrichment method is that when the specific organisms are found, they usually occur in abundance in one or more of the subcultures, thus saving much of the time which is taken up in testing a variety of "likely" colonies from the direct plates.

The practical value of telluric acid along with brilliant green is well illustrated by one of the cases recorded by Browning, Mackie, and Smith (Table VII).

TABLE VII
CASE M'I. (FAECES)

10 c.c. peptone-water medium containing—		Resulting growth in subcultures made after twenty-four hours' incubation at 37° C.
Telluric Acid 1: 1000	+ Brilliant Green 1: 10,000.	
0	0.1 c.cm.	Mainly inosite-fermenters + scanty colonies of <i>B. coli</i> .
	0.2 "	Mainly inosite-fermenters + scanty colonies of <i>B. coli</i> .
	0.3 "	Pure culture of inosite-fermenter.
	0.4 "	" "
	0.55 "	" "
0.33 c.cm.	0.1 "	Mixed growth.
	0.2 "	Almost pure <i>B. typhosus</i> .
	0.3 "	Pure <i>B. typhosus</i> .
	0.4 "	" "
	0.55 "	No growth.
0.5 "	0.1 "	Pure <i>B. typhosus</i> .
	0.2 "	" "
	0.3 "	" "
	0.4 "	" "
	0.55 "	No growth.
0.33 "	0	Pure growth of <i>B. Morgan</i> No. 1.
0.5 "	0	" "
Direct Plate Culture . . .		No <i>B. typhosus</i> found.

Thus the brilliant-green series yielded coliform bacilli and inosite-fermenters; the telluric-brilliant-green series yielded in three of the tubes pure *B. typhosus*. Telluric acid, in the absence of brilliant green, led to the appearance of a pure growth of an organism with the fermentative characters of *B. Morgan* No. 1. In the direct MacConkey plate no typhoid bacilli were found.

THE EXPERIENCE DERIVED FROM EXTENDED USE
OF THE METHOD OF ENRICHMENT IN FLUID
MEDIUM CONTAINING BRILLIANT GREEN AND
TELLURIC ACID BY INDEPENDENT WORKERS.

The results obtained in the past with other methods show how often workers who have originated a procedure, appear to develop a knowledge of the conditions essential to success, which they seem unable to communicate to others. Accordingly, if a method is to attain to practical importance it should work satisfactorily in independent hands. A number of observers have expressed a favourable opinion of the value of the present method (Dreyer, Stokes and Clarke, Leitch, Tidy and Dunn).¹ Certain others have reported unfavourably; thus Robinson condemns it, as has been already noted, apparently on the result of an experiment with artificial mixtures of pure cultures of *B. coli* and *B. typhosus*; Teague and Clurman conclude that the method is a failure since it does not give "as many positives as direct plating on Endo medium," but they do not bring forward any evidence to show that they have personally instituted a comparison between it and other methods such as their own more recent dextrose-bromoform-congo-red-bril-

¹ Some workers have reported that the use of brilliant green did not lead to the discovery of typhoid bacilli, but inquiry elicited the fact that they had not obtained positive results in their cases by any other method; it is scarcely possible to meet this form of objection except by the statement that we have never professed that brilliant green generates typhoid bacilli in faeces!

liant-green-gelatin *plus* brilliant-green-eosin-agar plate procedure. Krumwiede and Pratt in an earlier publication spoke favourably of the fluid enrichment medium, but later, along with McWilliams, they quote results in which it compares unfavourably with direct inoculation of the faeces on modified Endo's medium. An examination of their procedure shows that they used 0.1 c.cm. of a "moderately heavy suspension" of faeces (in a later part of their paper a 1 to 15 emulsion of solid faeces is specified) to inoculate each tube of brilliant-green fluid medium, whereas to inoculate each plate of the solid brilliant-green medium, which they themselves devised, they used only one and two loopfuls of the same faecal emulsion.¹ The amount of emulsion which they employed for the fluid medium was certainly excessive and would tend to overwhelm the antiseptic present; further, they made the reaction neutral to phenol-phthalein, *i.e.* markedly alkaline to litmus, which diminishes the differential efficiency of the dye; in our experience these points would explain their unsatisfactory results.

Rajchman and Western in an investigation of cases of "Bacillary Enteritis" state that they employed "a preliminary twenty-four hours' incubation of an emulsion of the faeces in a dilute solution of brilliant green with a subsequent plating of a few drops of the incubated fluid," but when tested on a series of fifty cases side by side with their routine

¹ For each specimen of faeces four of their brilliant-green agar plates and two Endo plates were inoculated.

procedure (inoculation of two plates of litmus-lactose-agar in succession) they were "unable to discover any difference in the results." It does not appear from the statements of these observers, however, how many of those cases yielded pathogenic organisms by their routine method, nor whether the positive cases showed typhoid-paratyphoid bacilli or dysentery organisms—the latter, of course, being unsuited for isolation by means of brilliant green, as we have pointed out.

Very extensive use of the method has been made by Stokes and Clarke in connection with a serious epidemic of typhoid fever among civilians in an area about to be taken over by British troops, also by Leitch at the laboratory of the Addington Park War Hospital, which is the final depot for soldiers convalescent from enteric and dysenteric diseases, and further by Tidy and Dunn; the results have been extremely favourable, as also have been those of Dunn and McNee, and McLeod (unpublished). This is especially striking since *only one tube of fluid medium was used for each specimen of faeces*, viz. 0·4–0·5 c.cm. of brilliant green 1 : 10,000 in 10 c.cm. of peptone water (other details of the procedures followed by these workers have been noted above). Stokes and Clarke examined 800 cases and found as follows :

Organism.	Total no. of cases in which found.	Found in direct plate.	Found by means of brilliant green.
B. typhosus . . .	15	15	14
P. paratyphosus B . .	11	3	8

Thus, their results demonstrate the special value of brilliant green for the detection of infections with *B. paratyphosus* B, since out of a total of eleven cases, eight were found by the enrichment method which had been missed in the direct cultures of the faeces on Endo agar; this is confirmed also by Dunn and McNee, and McLeod (unpublished). As Stokes and Clarke note, however, "this statistical result leaves out of the picture the relative ease of the two methods so far as finding the colonies is concerned. The balance from this point of view is strongly in favour of the brilliant-green method, in which it was common to get a practically pure plate of the typhoid-group organism."

Leitch employed for each specimen a single tube containing 1 in 200,000 of brilliant green, with the addition of telluric acid in the concentration which we recommended (1 in 25,000); 1023 specimens from 622 individuals were examined with the following results:

Method.	No. of specimens with "likely" colonies.	No. of specimens in which "likely" colonies were lactose fermenters.	No. of specimens from which cultures were carried to other sugars.	No. of specimens in which agglutination tests were necessary.	Actual positives.
Direct plating	373	59	314	161	17.
Brilliant green and telluric acid	120	39	81	45	21

The following important points in favour of the

enrichment method emerged from Leitch's tests: (a) In no single case was a pure growth obtained by direct plating; they were always mixed, but by the brilliant-green-telluric method there were pure growths of colourless colonies in sixty-two cases. When the growth on the indirect plate was mixed, the colourless colonies generally preponderated, and in all cases were easily detected. (b) In no single case by direct plating was the plate sterile; whereas 50 per cent. of plates by the indirect method showed no growth whatever. This means an enormous saving of time. (c) By the direct method the final test of agglutination had to be done in 161 cases, with a positive result, so far as typhoid-paratyphoid bacilli were concerned, in seventeen cases, while, on the other hand, in the case of the telluric-brilliant green method only forty-five cases had to be carried to agglutination, with positive results in twenty-one. The 622 individuals included two known typhoid carriers. Typhoid bacilli were picked out on all of five occasions in which the faeces of these carriers were examined by the brilliant-green method, and on four occasions by the direct plate. *B. paratyphosus A* was picked out on the single occasion by both methods. Paratyphoid *B* bacilli were isolated on fifteen occasions by telluric-brilliant-green and on twelve occasions by the direct plate; but this only gives one side of the picture, for it happened that no less than five paratyphoid *B* carriers were detected by brilliant green which were altogether missed by the direct method, whilst, on

the other hand, two carriers were found by the latter method and missed by the former.

Tidy and Dunn refer especially to their success in the isolation of paratyphoid bacilli by means of brilliant-green peptone-water enrichment and emphasise the value of the method for the recovery of *B. paratyphosus* A ; thus, in the examination of 200 cases they used 5 c.cm. of brilliant green 1 : 200,000 in peptone water and subcultured after twenty-four hours. *B. paratyphosus* A was isolated in fifty-one cases, in fifteen of which it was recovered by means of brilliant green when the control was negative ; *B. paratyphosus* B was isolated in sixty-four cases, eleven of which were negative by the control method. (The control method consisted in making a culture of the faeces in broth from which, after two or six hours' incubation, plate cultures were made on MacConkèy's medium.)

A further striking experiment by Benians illustrating the value of the method is quoted by Leitch. The urine of a carrier in which *B. paratyphosus* A and also *B. coli* could be demonstrated by direct plates was added to fifteen other urines in the proportion of 1 to 10. To a parallel series brilliant green was also added in a concentration of 1 in 250,000. After twenty-four hours' incubation plates were spread, with the result that in the series without the dye only one case yielded the paratyphoid organism, though *B. coli* were found in abundance on all plates, whilst by the interposition of brilliant

green the paratyphoid organism was found in nine cases in practically pure culture.

Both Stokes and Clarke and also Leitch have found that in certain cases the specific organisms were present in direct plates although they could not be recovered from the fluid brilliant-green cultures. The danger of missing the specific organisms by the enrichment method is, of course, greatest where only a single concentration of brilliant green is employed, as Mackie's results clearly show (see p. 131), although Stokes and Clarke cite a case in which the direct plate yielded *B. typhosus*, but cultures from four different dilutions of brilliant green alone were negative. This possibility has, however, never been lost sight of, and we have always recommended, in addition to the use of the enrichment medium, a direct inoculation from the faeces on a single plate of medium such as MacConkey's or Endo's.¹

As an offset against this, one must remember, as already noted, that *successful isolation of the specific organisms was secured by enrichment in brilliant green fluid medium from specimens in which they were originally so scanty that they could have been obtained on direct plates of solid medium only by obtaining over a thousand isolated colonies.* Dunn and

¹ Repeated examinations of three strains of *B. typhosus* isolated by the direct plate, but missed in the single brilliant green peptone-water tube (without telluric acid), showed that they were not highly susceptible to the dye (Nos. 500, 521, 551, see Table II, p. 103; for these we are indebted to Stokes and Clarke).

McNee¹ have reported, among others, three striking cases in which *B. typhosus*, paratyphosus A and B respectively, were isolated by means of the single brilliant green tube, although 2-3 direct plates on MacConkey's medium were negative in each instance. The following is a characteristic example:

Case P. W.:

(1) Two MacConkey's plates inoculated directly from the faeces. Six suspicious colonies from these plates investigated, all with negative results.

(2) Brilliant-green peptone-water tube inoculated at the same time as the direct plates. The subculture from this on a MacConkey plate showed twenty red colonies, all the rest being of a non-lactose fermenting type. Three of the latter picked at random and subjected to further tests proved all to be Paratyphosus B.

Douglas and Colebrook employed the brilliant-green method in a very thorough examination of a series of cases from the Mediterranean which were more or less convalescent at the time of observation. They used three concentrations, viz. $2\frac{3}{4}$, 4, and 6 parts of brilliant green per million parts of peptone water. Their results bear out strikingly the value of the method for the isolation of organisms of the paratyphoid group, and they also were able by means

¹ These workers have found (unpublished) that under military conditions blood cultures are extremely liable to be contaminated with streptococci, which are troublesome owing to the fact that they grow readily in subcultures on MacConkey's medium; but they can be excluded by inoculating brilliant green fluid medium from the blood culture, so enabling *B. typhosus* to be isolated.

of it to isolate many other non-lactose fermenting types of bacilli more frequently and more surely than by direct plating. This is an important confirmation and extension of Browning, Gilmour, and Mackie's observation as to the value of brilliant green for the recovery of certain atypical coliform organisms occasionally present in small numbers in faeces (see p. 102).

Teague and Clurman in their review of methods remark upon the results likely to be obtained from direct plating of faeces "properly performed." They quote elsewhere the estimate of Ficker and Hofmann (*Arch. f. Hyg.* 1904, 49, p. 229) that "1 : 300 represents the lowest proportion of typhoid that still gives positive results," but do not define their criterion of properly performed plating either as regards the number of isolated colonies to be attained, or the number of "likely" colonies which ought to be subjected to examination, when, as not infrequently happens, numerous pale colonies develop.

The measure of success which has been achieved by Stokes and Clarke, Leitch, and Tidy and Dunn in their series of cases numbering altogether over 1600, with a single concentration of brilliant green, speaks strongly for the value of the enrichment procedure and the ease of its application. The objection urged against the extended method is, of course, the time which it requires, but Leitch's observations show that so much labour is saved by diminution of the accessory procedures that an extension of the number of tubes of fluid medium might well be adopted without adding any additional burden of work as compared with the methods ordinarily in vogue. Thus, in Leitch's experiments

quoted above, the brilliant-green-telluric procedure was carried out by himself alone, whereas in the examination of the direct plates a staff of five was employed; to mention a single very important item, only forty-five organisms recovered by the brilliant green method required to be subjected finally to agglutination tests, whereas 161 organisms isolated from the same cases by direct plating had to be examined for agglutination.

We ourselves have quite definitely observed that a series of tubes may be required in order to obtain a positive result, *e.g.* in the following instance (Browning and Thornton):

Solid faeces obtained by enema—one loopful emulsified in 5 c.cm. saline: of this one large loopful was used to inoculate each tube of 10 c.cm. of enrichment medium: (a) series containing brilliant green (1:10,000) alone, 0·1, 0·2, 0·35, 0·5, 0·7 c.cm.; (b) ditto, with, in addition, telluric acid 1:25,000. Result of subcultures made after twenty-four hours at 37° C. Series (a) only coliform bacilli; Series (b) *pure culture of B. typhosus in the highest tube; in all the others only coliform organisms.* The direct plate culture on MacConkey's agar showed nothing resembling *B. typhosus* among over 100 isolated colonies. (It is striking how small an amount of faeces was used to inoculate the enrichment tubes in this instance.)

It might be argued that the positive tube was the only one in which the inoculation material contained a viable typhoid bacillus to begin with, but our experimental results with mixed cultures in which abundant typhoid bacilli were present in the inoculation material (Table VI) prove that such cannot be the complete explanation. Also we have seen the faeces from a case yield a pure culture of

B. typhosus after enrichment with brilliant green 1 : 714,000 ; whereas with a concentration of 1 : 227,000 there resulted only scanty colonies of *B. typhosus* along with *B. proteus* and coliform bacilli (Browning, Gilmour, and Mackie). Robinson and Rettger, who have confirmed the efficacy of brilliant green in fluid medium, state that "the importance of employing the dye in varying dilutions is very apparent." Recently Mackie, working in the East, where Paratyphoid A and B were the prevailing organisms, has amply confirmed this point and has shown that if the greatest number of positive results is to be obtained it is essential to use a series of amounts of brilliant green as originally recommended. He employed for each specimen of faeces three 10 c.cm. peptone-water tubes containing 0·25, 0·4, and 0·6 c.cm. brilliant green 1 : 10,000 ; a large loopful (0·4 cm. diameter) of a dense uniform emulsion of faeces was used for inoculation, or in the case of diarrhoeic faeces 3 or 4 loopfuls, and subcultures were made after seven to twelve hours' incubation at 37° C. The following table summarises his results and shows in the clearest fashion that the so-called " optimum " (0·4–0·5 c.cm.) amount of brilliant green recommended by other workers failed in half the cases to give a positive result ; in one instance the specific organisms were obtained only with a smaller amount of antiseptic and in two others only with a larger dose, although in one of the latter the " direct " plate was positive.

Amount of Brilliant Green 1 : 10,000 per 10 c.cm. medium.	Cases with B. paratyphosus A				Cases with B. paratyphosus B	
	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.	No. 6.
0.25 c.cm. . . .	++	—	—	—	—	++
0.4 c.cm. . . .	—	+	—	+	—	++
0.6–0.7 c.cm. . .	—	++	+	—	+	++
Control : Result of “direct” plate culture	—	—	2 colonies	—	—	a few colonies

++ = Practically pure growth of specific organism.
 + = Mixed growth with abundant specific organisms.
 — = Specific organisms not found.

Thus a positive result may depend entirely on adjusting conditions so as to favour the proliferation of the specific organisms. Hence we believe the fact has been demonstrated that the employment of a series of doses of the reagents is important, and that *with the expenditure of a given amount of time* this procedure, in addition to direct plating on such media as MacConkey's or Endo's, is likely to yield the best return of positive results in typhoid and paratyphoid infections. We should suggest as a minimum either (a) brilliant green 1 in 400,000 and 1 in 200,000 with and without telluric acid 1 in 25,000, or (b) if brilliant green be employed alone, concentrations of 1 : 400,000, 1 : 250,000, and 1 : 150,000.

Where it is necessary to curtail labour to a minimum, compatible with efficient method, direct plating and the inoculation of a single tube of brilliant-green peptone water may be practised as a

preliminary; at the same time the specimen of faeces should be preserved in dilute glycerine according to Teague and Clurman's procedure and thus it will remain available for further examination by the brilliant green telluric method should the results of the preliminary cultures prove negative (see p. 138).

In view of the evidence here adduced in favour of the use of a fluid medium containing brilliant green and telluric acid for the isolation of typhoid and paratyphoid bacilli, it seems justifiable to suggest that those who bring forward further new methods, should in the first place institute comparative tests between their procedures and that which we have described before claiming that their improvements excel all that has hitherto been achieved. Further, the brilliant-green method must be tested under the conditions which have been shown to be most suitable for its efficient working, and in making comparisons regard must be had to the relative simplicity of the methods examined and to the time absorbed by each.

THE EXAMINATION OF URINE AND OF CONTAMINATED WATER FOR TYPHOID AND PARATYPHOID BACILLI

The value of bacteriological examination of the *urine* in cases of typhoid-paratyphoid infections has not been so extensively recognised as it ought to be. When the specific organisms are scanty, centrifugalising and cultivating from the sediment prob-

ably does not represent the best method. It is preferable to incubate comparatively large volumes of the urine. Morishima and Teague find, however, that certain urines inhibit the growth of *B. typhosus*, but that this inhibition is overcome by adding two volumes of broth to one of urine and then incubating overnight. Owing to the liability of specimens of urine to contamination, brilliant green should be used in the mixture (see Benians' striking results, p. 126).

When examining contaminated specimens of *water* for typhoid or paratyphoid bacilli, it is well known that the chances of success in the past have been practically nil. It is suggested that the following procedure should be tried—To a large volume of the water sufficient alum and carbonate of soda are added to produce within a quarter of an hour an abundant precipitate (7 c.c. each of a 5 per cent. solution of potash alum and a 1.5 per cent. solution of sodium carbonate per litre of water, have been found suitable), which carries down the greater part of the organisms. When this has settled the supernatant fluid is pipetted off and the sediment concentrated if need be by the centrifuge. The sediment is then used to inoculate a series of flasks containing varying strengths of brilliant green in peptone water, with and without telluric acid. In this way a much larger volume of contaminated fluid could be dealt with than is likely to be manageable by any other method.

SUMMARY

It has been proved both by the use of artificial mixtures and also by the examination of the faeces of extensive series of patients, totalling over 1600 and involving over 160 positives, by a number of independent observers, that :

(1) the isolation of typhoid and paratyphoid bacilli can be greatly facilitated by employing, in addition to direct-plate cultures on media such as MacConkey's or Endo's, the method of enrichment in a simple peptone-water medium containing the differential antiseptics brilliant green and telluric acid.

(2) Since the adjustment of conditions necessary to favour the proliferation of the specific organisms in a mixture with coliform types, as in faeces, depends on a number of variables, which cannot be standardised, it is advisable—one might well say essential—to use a series of tubes of the enrichment medium containing graded amounts of antiseptic in order to obtain the maximum number of positive results. A single concentration of brilliant green (1 to 200,000) with or without telluric acid 1 to 25,000 has been employed with great success, especially for the isolation of *B. paratyphosus* B; it is certain, however, that this single-tube method will not yield the maximum number of positives.

(3) A specially favourable feature of the method is that in positive cases a practically pure culture of the typhoid-paratyphoid organism is frequently

obtained, so that there is a great saving of the time ordinarily spent in testing a number of likely colonies.

(4) It is suggested that by utilising the glycerin preservation method for faeces, a direct plate and a single brilliant-green fluid culture may be made in the first instance; should these be negative the specimen will then still be available for the extended procedure with a series of doses of brilliant green and brilliant green *plus* telluric acid. This would permit of the greatest saving in labour, compatible with a thoroughly efficient examination.

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ADDENDUM

THE ISOLATION OF TYPHOID AND PARATYPHOID BACILLI FROM FAECES TREATED WITH GLYCERIN

A METHOD OF PRESERVING FAECES FOR DELAYED EXAMINATION

Teague and Clurman found that in the case of faeces containing typhoid bacilli, these organisms could be isolated from emulsions of the faeces in dilute glycerin which had been allowed to stand for several days, whereas similar emulsions made up with saline alone soon failed to yield *B. typhosus* in cultures. This method of preserving the faeces for delayed examination was accordingly recommended.

Procedure.—A small amount of faeces, as soon after evacuation as possible, is emulsified by stirring up with about double the volume of 30 per cent. glycerin in 0.6 per cent. NaCl solution.

Benians¹ has confirmed this observation of Teague and Clurman and has further extended the observation to cases of Paratyphoid A and B infections. He investigated twelve carriers, two of *B. typhosus*, one of *B. paratyphosus* A, and nine of *B. paratyphosus* B, all of which yielded colonies of the specific organism on direct MacConkey plates. About 5 grams of faeces were emulsified in 10 c.cm. of glycerin-saline, and as a control a similar emulsion was made up in saline without glycerin. Every day a measured quantity of the

¹ Benians, *Lancet*, 1918,

supernatant fluid was removed and spread over one well-dried MacConkey plate in each case. The plates were examined after twenty-four hours' incubation at 37° C. In one set of experiments the emulsions were left at room-temperature (16° C.) in the laboratory, in another test they were kept at 37° C. throughout.

The specific organisms were always identified by agglutination with potent antisera. The results were as follows :

Age of emulsion (kept throughout at 16°C.).	Number of cases in which the specific pathogenic organisms were isolated from the emulsion in	
	Glycerin-saline.	Saline alone.
1st day	12	12
2nd „	12	12
3rd „	10	10
4th „	10	6
5th „	10	3
6th „	10	0

Thus the preservation of the specific organisms by means of glycerin is clearly proved. The two cases which fell out on the second day were a *B. typhosus* and a *B. paratyphosus* B infection, both of which were to begin with very scanty in number.

In a number of the cases non-specific organisms which failed to ferment lactose were also present. It was found that glycerin had also the effect of preserving these ; whereas in the saline emulsion the lactose fermenting coli types rapidly overgrew the others.

The experiments in which the effect of a warm climate was imitated by keeping emulsions at 26° C, showed that :

(1) In the case of one specimen each of faeces containing *B. typhosus*, *B. paratyphosus* A, and *B. paratyphosus* B, glycerin emulsions kept at 26° C. yielded as many white colonies as emulsions kept at 16° C. for the first three days, by the fourth day a slight increase in red colonies occurred in the 26° C. specimens.

(2) On comparing a glycerin and a saline emulsion of faeces heavily infected with *B. paratyphosus* B, kept at 26° C., the number of non-lactose fermenting bacilli was maintained in glycerin—the number of coliform organisms falling somewhat—whereas in the plain saline emulsions the lactose fermenters increased enormously and the paratyphoid bacilli fell rapidly in numbers, disappearing entirely by the fourth day.

Conclusion.—The glycerin preservation of faeces represents an important addition to methods in the examination of faeces for typhoid-paratyphoid bacilli. This method should be employed whenever examination is to be delayed, *e.g.* when the specimen has to be transmitted for a distance, or if it is desired to delay the extended brilliant-green telluric-acid method until it is seen whether the simple direct plate and one tube green culture fail to give positive results.

(Unfortunately no examination of *B. dysenteriae* infections has been made.)

VII

THE ISOLATION OF B. DIPHTHERIAE BY MEANS OF A SIMPLE MEDIUM CONTAIN- ING TELLURIC ACID

BY J. F. SMITH

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Paper incorporated

J. F. Smith, *Journ. of Path. and Bact.*, vol. xix. 1914, p. 122.

THE isolation of B. diphtheriae from a mixture of organisms, such as is found on swabs from the throat, may present considerable difficulty. Experiments have been carried out with a view to finding substances which would inhibit the growth of contaminating organisms without affecting B. diphtheriae. A large number of compounds, chiefly basic dyes and salts of the metalloids, have been tested, with the general result that B. diphtheriae is among the organisms most susceptible to the inhibitory action of these bodies. It was found, however, that towards telluric acid and its salts, B. diphtheriae, and the group of diphtheroid bacilli in general, are considerably more resistant than the

majority of pathogenic organisms, including staphylococci and the coli-typhoid group.

Potassium tellurate along with calcium bimalate was recommended for the isolation of *B. diphtheriae* by Conradi and Troch, who included these salts in a medium similar to that of Loeffler. This does not seem to have come into general use ; partly, perhaps, because in several works Kalium "tellurosum" is mentioned instead of "telluricum." I have found that the differential antiseptic action of tellurite is less marked than that of tellurate. It is not clear from Conradi and Troch's publications whether they recognised the differential antiseptic action of telluric-acid salts as favouring the growth of *B. diphtheriae*. Potassium tellurate seems to have been included in their medium rather as an indicator, which when reduced by acid formed from glucose by *B. diphtheriae* caused the colonies to appear black, although on one occasion they refer to "enrichment" of a positive throat culture by growth on their medium. A disadvantage which attaches to the medium of Conradi and Troch is that certain strains of streptococci occurring in the throat form on it a moist confluent growth, and this obscures the colonies of *B. diphtheriae*.

A simple serum-agar medium containing telluric acid has been devised, which has given better results in the isolation of *B. diphtheriae* and diphtheroid bacilli than the media either of Loeffler or of Conradi and Troch. The constitution of this medium is as follows :

Sheep serum, sterilised by heating on several occasions for 1 hour at 57° C.	5 c.cm.
1 per cent. telluric-acid solution in distilled water ¹	0.9 „
Peptone-water agar (roughly neutral to litmus)	to 100 „

The serum is added to the melted agar at a temperature of 50° to 60° C. The medium may subsequently be heated at 57° C. for several hours without injury to its properties. On this medium, *B. diphtheriae* forms moderately large, opaque, white colonies after twenty hours' growth at 37° C. Such other organisms as grow generally show colonies darkened by reduced tellurium. Streptococci resist telluric acid well, but are not a serious contamination, as they form minute discrete colonies. *Diphtheria bacilli* grown on this medium show the characteristic granules when stained by Neisser's method, not quite so well marked as in culture on Loeffler's serum, but better than on Conradi and Troch's medium. All diphtheroid organisms appear to grow well on telluric-serum agar medium (*e.g.* *B. Hofmann*, *ozaena diphtheroids*, etc.). The amount of serum employed seems to constitute an optimum, since when a larger proportion of serum was present in the medium the differential action was less marked, other organisms being then more resistant to telluric acid, and with smaller amounts of serum

¹ Originally 1·5 c.cm. of 1 per cent. solution of potassium tellurate, less than a week old, was employed; on standing, the tellurate solution gradually loses its antiseptic action, whereas telluric-acid solution, if sterilised by heat, keeps indefinitely.

the growth of *B. diphtheriae* was not sufficiently vigorous. The reaction also has some effect since a high degree of alkalinity of the medium tends to counteract the effect of the telluric acid.

The following is an illustrative experiment undertaken to show how this medium compares with those of Loeffler and of Conradi and Troch for the isolation of *B. diphtheriae* :

To a series of tubes each containing 3 c.cm. of a fairly turbid emulsion, in 0·85 per cent. NaCl solution, of a negative throat culture, containing mainly staphylococci and streptococci, a very thin emulsion of a twenty-four hours' growth on agar of a recently isolated strain of *B. diphtheriae* was added in the following amounts—0·005, 0·01, 0·02, 0·03, and 0·1 c.cm. A loopful from each tube was stroked on plates of each of the three media, and these were incubated for twenty hours at 37° C. On Loeffler's serum *B. diphtheriae* could not be detected microscopically even in the strokes from the tubes containing the highest concentrations of *B. diphtheriae*, while on the other media diphtheria bacilli were found in the cultures from the lowest concentration of *B. diphtheriae*. On the telluric-serum agar *isolated* colonies could be picked out on all the strokes, but none could be found on the Conradi and Troch plates, even up to the highest concentration. The naked-eye appearance of the diphtheria colonies, as opaque white discs without any suggestion of a brown coloration after twenty hours at 37° C. or even later, enables them to be recognised

with comparative ease even when other organisms are present on the telluric-serum agar.

Such results as the following indicate also that the telluric-serum agar medium will present advantages over other media for the recognition by microscopic examination of the presence of *B. diphtheriae* in certain cases, depending probably on the character of the associated organisms :

To a series of tubes each containing, as in the former experiment, 3 c.cm. of an emulsion of a negative throat culture on Loeffler's serum, *B. diphtheriae* emulsion was added in the following amounts : 0·035, 0·05, 0·1, 0·25, and 1·0 c.cm. Strokes were made on plates and incubated as before.

Medium	0·035	0·05	0·1	0·25	0·5	1·0
Loeffler . . .	—	—	—	+	+	+
Conradi and Troch . . .	—	—	—	—	—	+
Telluric-serum agar . . .	—	+	+	+	+	+

isolated colonies.

+ signifies recognition of *B. diphtheriae* microscopically.

An examination of cultures from swabs of the throats of fifty cases suspected of diphtheria (including some contacts) gave the following results :

	Loeffler.	Conradi and Troch.	Telluric-serum agar.
Positive . . .	8	6	10
Negative . . .	42	44	40

In no case was a negative result obtained on telluric-serum agar and a positive on either of the other media.

The greatest advantage of this medium consists in the ease with which organisms belonging to the diphtheria group can be isolated in pure culture for further investigation. It is a matter of common experience among those who have seriously approached this problem that the isolation of *B. diphtheriae* in pure state is frequently very tedious. The further investigation of the relationships of the so-called non-toxic or avirulent true diphtheria bacilli to the disease, a question still quite obscure, will be facilitated by the use of such a relatively simple and convenient selective medium as telluric-serum agar. It is important that when an apparently pure colony of *B. diphtheriae* has been obtained on any medium, it should be subcultured several times, preferably on telluric-serum agar, from a single colony on each occasion, in order to exclude the liability to latent contamination.

In the investigation of the diphtheroid bacilli occurring in the nasal crusts in cases of ozaena, I have found this medium of great value. On Loeffler's medium the diphtheroid organisms are almost invariably overgrown by the *B. mucosus ozaenae* of Abel and by staphylococci, but on telluric-serum agar they are easily isolated, and are in many cases almost the only organisms to grow.

The medium here described would also prove of service for the isolation of streptococci.

VIII

THE SELECTIVE ACTION OF THALLIUM ACETATE IN SUPPRESSING *B. PYOCYANEUS* AND ORGANISMS OF *PROTEUS* TYPE (*UROBACILLUS SEPTICUS*) AND OF THORIUM NITRATE IN PERMITTING GROWTH OF *B. MALLEI*

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OWING to their active growth on ordinary media and their high resistance to most inhibitory agents, *B. pyocyaneus* and *Urobacillus septicus*¹ when present tend to outgrow all other organisms in cultures, *e.g.* streptococci, so that the isolation of the latter is impossible. Observations have shown that these organisms can be suppressed, or so limited

¹ *Urobacillus septicus*, an organism of proteus type, occurs frequently in infections of the urinary tract, ear, etc.; it is a Gram-negative bacillus which is most readily identified by its *spreading* growth; thus if the centre of a plate of medium is inoculated with this organism, growth in the form of a semi-transparent film (much like *B. pyocyaneus* but without pigment) proceeds within twenty-four hours at 37° C. over the whole surface.

by the addition of thallium acetate to agar medium that, at most, local, discrete colonies of urobacillus are formed, while at the same time many streptococci grow vigorously (see Table, p. 149). Thus the use of thallium acetate appears to offer the best prospect for the isolation of streptococci when present along with *B. pyocyaneus* and *B. proteus* types which exhibit spreading growth.

Thorium nitrate is characterised by its discriminating action in favour of *B. mallei* as compared with other organisms liable to be found in glanders lesions. Two different strains of *B. mallei* have exhibited this character.

In the table an experiment is shown in which thallium acetate, thorium nitrate, and telluric acid were employed simultaneously with the same cultures, so that the selective effects of the different compounds are thus proved to be real and not merely apparent.

Organism.	10 c.cm. peptone-water agar, neutral to litmus, containing:									
	Tballium acetate 1 per cent.				Thorium nitrate 1 per cent.				Telluric acid 1 per cent.	
	0.1 c.cm.	0.5 c.cm.	1.0 c.cm.	2.0 c.cm.	0.6 c.cm.	1.0 c.cm.	1.5 c.cm.	2.0 c.cm.	0.6 c.cm.	3.0 c.cm.
Staphylococcus aureus.	+++	++	++	+	++	++	++	++	++	++
Streptococcus B. mallei	++	++	++	++	++	++	++	++	++	++
B. pyocyaneus	++	++	++	++	++	++	++	++	++	++
Urobacillus septicus	++	++	++	++	++	++	++	++	++	++

Control cultures on agar without antiseptic all + + + +.

+ + + = abundant growth.

+ + = slight inhibition.

+ = marked inhibition.

IX

ON THE POSSIBILITY OF EMPLOYING ULTRA-VIOLET RADIATION AS A MEANS OF DISCRIMINATING BETWEEN OR- GANISMS AND FOR THEIR SELECTIVE ISOLATION

WITH A DESCRIPTION OF A METHOD OF DETER-
MINING THE EFFECTIVE BACTERICIDAL PORTION
OF THE ULTRA-VIOLET SPECTRUM

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Paper incorporated

Browning and Russ, *Proc. Roy. Soc., B*, vol. 90, 1917, p. 33.

BACTERIA, pathogenic and otherwise, are killed by exposure to ultra-violet radiation. If there were a wide range of susceptibility of these organisms, then a method based upon this phenomenon might serve to distinguish one organism from another. There are two considerations bound up with any such method. Two different organisms such as *B. coli* and *B. typhosus* when exposed to the composite ultra-violet radiation from an arc may require differ-

ent exposures in order that a lethal effect may be obtained ; such differences if they existed would be classed as differences of *vulnerability*. If we now arrange that the organisms are exposed to the ultra-violet spectrum in such a way that the subsequent lethal action can be observed over a scale of wave-lengths, then if the range of action is different in the case of two organisms exposed to the same source of radiation such differences would be classed as differences of *susceptibility*.

A *prima-facie* consideration of the alternatives as a method of separation or identification of bacteria inclines one to the view that the difference of susceptibility would be a safer guide than that of vulnerability.

In what follows a method is described (which is similar to that adopted by Barnard and Morgan) by which it is possible to determine definitely what portion of the ultra-violet spectrum is especially effective in germicidal action and the wave-length of the radiation at which such action practically ceases.

The Quartz Spectrometer (Design C) made by Messrs. Hilger is so constructed that photographs may be obtained ranging from wave-lengths 7000–2100 A.U. ; this region of the spectrum is spread over a length of about 19 cm. (it varies slightly with each instrument).

The radiation emitted by an arc of pure tungsten when focused on the slit of the instrument and received on the photograph plate is seen to consist

of a series of bright lines very closely packed together extending as far as 2100 Ångström Units. Thus it is a very suitable source of intense ultra-violet radiation over a wide range.

In order to determine what part of this radiation exerts germicidal action the following procedure was employed. A glass plate, similar to the photographic plate used in this instrument, was coated with a thin layer of nutrient agar; then over this surface a thin layer of a living bacterial emulsion (*Staphylococcus pyogenes aureus*) was painted. The plate thus inoculated was then placed in the carrier of the instrument and the shutter opened to allow the radiation from the arc to fall upon a narrow strip of the bacterial film. After an appropriate exposure the plate was removed from the spectrometer and then incubated at 37° C. for forty-eight hours in order to see, by the resulting growth, what effect had been produced upon the organisms by the various constituents of the beam. In the illustration, Fig. 3 shows the germicidal effect of the rays for three different times of exposure of the organisms, viz. 6, 12, and 24 minutes. The radiation utilised, as shown by its action on a photographic plate (top strip in Fig. 4), is seen to extend right across the bacterial film; the germicidal action is, however, restricted to a region which ranges from a wave length of 2940 A.U. to about 2380 A.U.

It is interesting to observe that the organisms appear to be stimulated in their growth in the

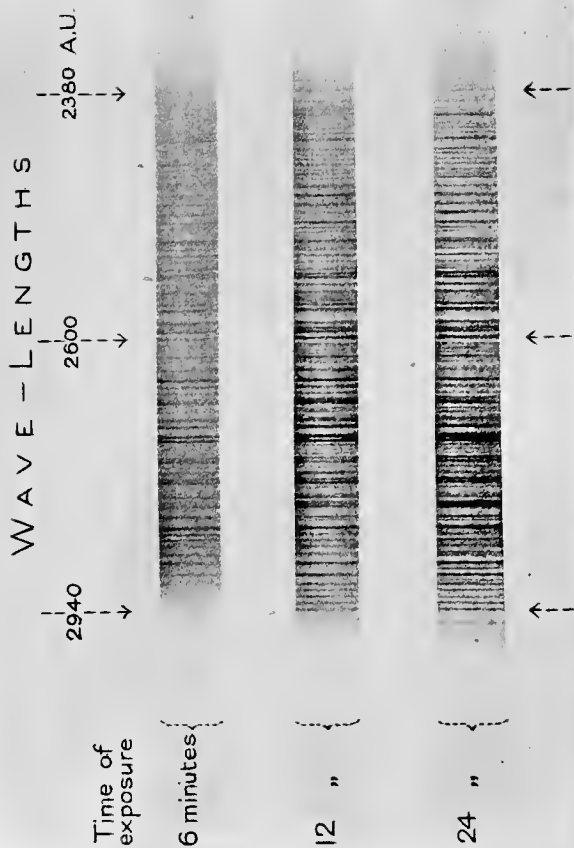


FIG. 3.

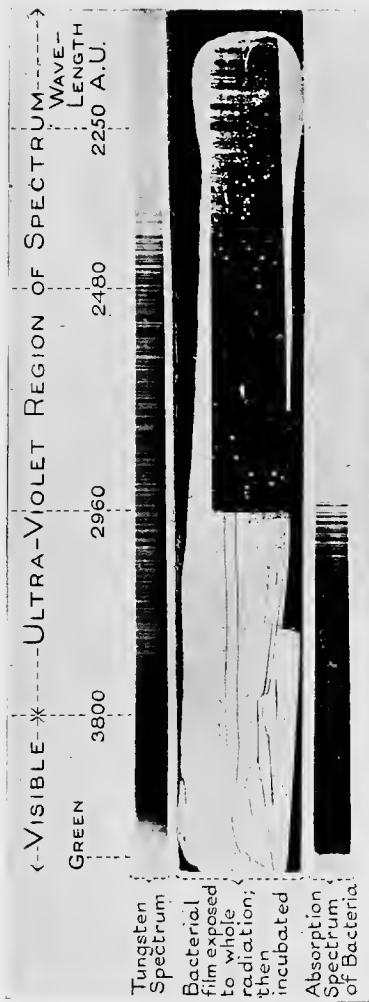


FIG. 4.

region just between two portions which had been exposed to the full radiation; photographs taken with long exposures always show the presence of scattered radiation. The increase of bacterial growth was confirmed microscopically, but it is not necessarily due to the scattered radiation.

The effect of prolonging the exposure from six to twenty-four minutes is to increase somewhat the range of lethal action, but only to a comparatively slight extent. A more prolonged exposure brings out prominently the region of the spectrum at which germicidal action practically stops. In order to increase the amount of radiation reaching the bacterial film, the slit was widened; this had the effect of causing the lines of the spectrum to overlap sufficiently to form a practically continuous band of radiation. The procedure followed was otherwise identical with that which has just been described; the bacterial film was first exposed to the whole range of spectral lines, then incubated to see what effect had been produced upon the organisms by the various constituents of the beam.

The central strip of Fig. 4 shows the result of a prolonged exposure (nearly four hours) of the organisms to the tungsten radiation. It will be seen that germicidal action occurs throughout a region of wave-lengths extending from about 2960 to 2100 A.U. The most striking feature is the sharp line of demarcation occurring in the region of the former wave-length. Ether vibrations of wave-

length 2960 A.U. have a marked germicidal action ; an increase of 1 or 2 per cent. in this wave-length is, practically speaking, sufficient to bring one to a region of the spectrum devoid of such germicidal action.

The existence of this sharp line of demarcation strongly suggested that selective absorption was playing some part. This was put to the test by examining the absorption spectrum of a suspension of the organisms which were used in the experiment just described. A small quartz vessel was filled with the bacterial emulsion and placed in front of the slit of the spectrograph ; a series of photographs was then taken of the radiation transmitted through the emulsion in which the exposure ranged from five seconds to five minutes and practically identical records resulted. The bottom strip Fig. 4 (exposure of two minutes) shows that the region of wave lengths absorbed by the bacterial emulsion corresponds almost exactly with that portion of the spectrum which has marked germicidal action.

THE GERMICIDAL ACTION UPON VARIOUS ORGANISMS

The method which has been described to determine what portion of the ultra-violet spectrum is responsible for germicidal action is well adapted for comparative tests of the effects of these rays upon different organisms.

BACILLUS COLI COMMUNIS AND BACILLUS TYPHOSUS

To determine the relative susceptibility of these

organisms, emulsions from agar cultures were made and spread over a surface of agar which took the place of the photographic film as already described. A central strip about 3 cm. wide running the whole length of the plate was separated from the rest of the agar by two glass strips a few millimetres in width. The central strip was painted over with the emulsion of one of the organisms and the rest of the agar with that of the other organisms. The plate was then placed in position in the camera, and the length of the slit adjusted so that the radiation from the arc illuminated both surfaces over which the organisms were spread, one of the glass strips lying exactly along the middle of the exposed area. In this way the two organisms were exposed to radiation of identical character for the same time; after a suitable exposure the camera attachment was racked down so that the second glass strip was in the centre of the exposed area and another exposure made. The agar plate was removed and incubated overnight; the resulting growth on the plate showed which organism had been more affected by the rays.

A series of observations ranging from 5–10–20–30 minutes showed that *B. typhosus* was rather more affected than *B. coli*; this difference in susceptibility was small, but quite definite for each of the exposures: for an exposure of five minutes, a lethal effect was obtained on the *B. coli* over a range of wave-lengths 2960–2450 A.U., for the *B. typhosus* the range was 2960–2400 A.U.; when the exposure was lengthened to thirty minutes the lethal effect

on *B. coli* ranged from 2960–2200 A.U. and that of *B. typhosus* from 3000–2100 A.U. Such small differences could hardly serve as a sure method of differentiation of these organisms.

MENINGOCOCCUS AND THE ACID-FAST BACILLUS (TIMOTHY GRASS B)

The susceptibility of these organisms to the rays in question was compared directly with staphylococcus by the method detailed above. (In the experiments in which the meningococcus was compared, "tryptagar" was employed as the medium.) After suitable exposures the agar plates were incubated and the range of wave-lengths over which a lethal action was subsequently observed was measured for the different organisms. The meningococcus was found to be slightly more susceptible than the staphylococcus; exposure of these two organisms to the same radiation for the same time (twenty minutes) resulted in a lethal action upon the meningococcus over a range 2960–2240, the range for staphylococcus being 2960–2320 A.U.

In the case of the acid-fast bacillus the result was reversed, the staphylococcus being slightly more susceptible than the acid-fast bacillus.

It is thus seen that many of the commoner pathogenic organisms have a range of susceptibility towards ultra-violet radiation which is not markedly different one from the other. The small differences which do exist in those which we have examined

would hardly serve as a reliable basis for the differentiation of one organism from another or for the separation of any particular species from a mixture of organisms.

REFERENCE

Barnard and Morgan, *Proc. Roy. Soc.*, vol. 72, 1903, p. 126.

X

THE CHEMISTRY OF ANTIBODY REACTIONS, WITH CONSIDERATIONS ON THEIR PRACTICAL APPLICATION

BY C. H. BROWNING

With which is incorporated Browning, *Brit. Med. Journ.*, vol. i.
1915, p. 239.

THE RELATIONSHIP BETWEEN IMMUNITY AND IMMUNITY (ANTIBODY) REACTIONS

ACQUIRED insusceptibility to infective disease if of any considerable degree of magnitude, such as will possess value in conferring notably enhanced protection on those exposed, is practically always produced specifically. This is effected either (1) as a consequence of submitting the individual to a modified attack of the disease by bringing the causal agent into direct contact with his tissues (in the form of "attenuated" virus, as in smallpox vaccination, or of dead cultures, *i.e.* "vaccines," as in antityphoid inoculation), whereby active immunity results, or else (2) as the result of conferring passive immunity, by introducing into the tissue fluids serum of an animal which has been rendered actively immune, *e.g.* in the treatment of diphtheria and the prophy-

laxis of tetanus. Now the rationale of these procedures depends on the fact that when infection occurs, *i.e.* when some living organism obtains a foothold in the tissues and produces harmful effects, the body of the host usually does not play merely a passive rôle, but undergoes alterations in its metabolism, which often result in the subjection and termination of the infective process. If the individual is again subsequently exposed to the same infection it is frequently found that a dose of the infective agent, which previously would have been able to produce the disease, now causes no ill effects; thus he has been rendered relatively immune. The strict proof of this fact of immunisation has been worked out experimentally where the dose of infective agent or its toxic products could be precisely regulated; it has also been arrived at statistically, as when large numbers of human beings have been subjected to Jennerian vaccination or antityphoid inoculation or to preventive immunisation with tetanus antitoxin, and the incidence of the disease has in consequence been greatly reduced (see p. 267).

The conclusion is justified that acquired immunity depends on changes excited in the living body by specific products of the infective agent. In the hope of arriving at a more detailed explanation of this phenomenon the behaviour of the tissues and body fluids of immunised animals has been extensively investigated since the date of Nuttall's classical observations, with the result that it has been shown

beyond all doubt that the body fluids of an immunised animal frequently possess properties, either qualitative or quantitative, which the susceptible one lacks; these properties are comprised in the term *antibodies*. The paramount importance of antibodies in certain cases is proved by the phenomenon of *passive immunity*, whereby insusceptibility to a disease, *e.g.* diphtheria or tetanus, is conferred on a normal individual merely by injecting into his tissues the serum of one which has been rendered actively immune in the fashion described above. It has been found that similar antibodies are generated by the introduction into the living body of a variety of substances which have only two properties in common, *viz.* (1) they are all proteins—for exceptions to this statement see p. 167—and (2) they are foreign to the normal tissues of the animal which reacts to them by the production of antibodies; they are classed generally as *antigens*. Antibody and antigen are terms each of which is explicable with respect to the other, and they are identified usually by the results which follow when they operate in conjunction, thus giving rise to antibody or immunity reactions. In certain cases there seems to be no doubt that immunity depends on a particular antibody, *e.g.* diphtheria toxin is rendered inert by antitoxin, and the fact that this neutralisation occurs *in vitro* shows that, in this instance at least, immunity may be wholly dependent on “humoral” constituents.

Unfortunately, wide generalisations regarding im-

munity have been founded on the result of a relatively small number of instances in which clearly cut experimental results of this and similar nature have been obtained. Thus, one school of investigation, represented by Ehrlich, has theorised regarding immunity on the basis of the properties of the tissue fluids, while Metchnikoff and his pupils have concentrated their attention on the behaviour of the cellular elements, some of which must, of course, be the source from which important constituents of the body fluids are derived. Again, it has been the result of the work of Wright and Douglas to bring into prominence the fact that the cells and the tissue fluids may co-operate in the case of phagocytic action. But, while certain manifestations of immunity have lent themselves to fairly complete experimental analysis, there has always been the danger of assuming that they fully explained immunity. Although there has been great inducement to embody generalisations in the form of attractive theories of immunity, well balanced, critical minds have felt that the complexity of biological phenomena could not, at least in the present time, be summed up in any general formula. It appears to be considerations of such a nature which have restrained Bordet and also Muir from basing any theories of far-reaching application on their fundamental observations.

Except in restricted instances it is probable that the known immunity reactions do not completely account for acquired immunity—not to mention

natural insusceptibility—and that many of the immunity reactions with which we are acquainted represent in great likelihood only results and side effects of the processes which underlie immunity. Immunity may exist without them, as when an animal which has been rendered actively immune to anthrax bacilli, and has then been inoculated with these organisms, continues to harbour living virulent bacilli in its circulation for many days without manifesting signs of disease. No immunity reaction can be demonstrated here, and yet the animal is immune! Again, there is little doubt that a degree of acquired immunity to typhoid fever persists for a long period after an attack or after prophylactic inoculation and is still effective at a time when antibodies have disappeared from the blood; similarly it has been shown that in animals immunised with pneumococci there may be a high degree of active immunity with practically no antibodies in the serum (Swift and Kinsella). Therefore, the antibody content of the blood does not necessarily gauge the immunity. Further, it is significant that a certain duration and intensity of action of the infective agent may be required in order that antibodies may be generated; thus, recovery may occur from an acute infection so rapidly that antibodies do not appear in the blood. This most important observation, which hitherto has attracted little attention, we owe to Macgregor in the case of patients with acute meningococcal meningitis terminating in rapid recovery, and it

points clearly to the possibility that antibodies may on occasion be subsidiary phenomena in the processes on which immunity depends.

The antibody is thus a frequent and important accompaniment of immunity and sometimes the cause; but it must always be borne in mind, firstly, that immunity may exist without our being able to demonstrate any of the recognised antibodies, and secondly, that where an antibody can be demonstrated it may be the participator in some side reaction not essentially concerned in protection against the infection. While antitoxic and bactericidal action undoubtedly contribute to the production of immunity, agglutination until recently was regarded as having little value in this respect. Carroll Bull, however, has shown that in the treatment of experimental pneumococcal septicaemia in rabbits by means of antiserum, clumping of the organisms in the blood is the first occurrence, and he advances the view that protection against the infection is determined by the presence of the agglutinins. A corollary of the greatest importance is that under these circumstances the treatment should take the form of repeated small doses of serum; large doses are detrimental, since, if given to begin with, they cause the formation of large clumps of cocci, which accumulate chiefly in the vessels of the lungs and impede the circulation; again, large doses following small ones are also harmful, as the consequence is destruction of the pneumococci under conditions which give rise to

intoxication. Such observations open up entirely new prospects in the field of serum therapy.

For practical purposes immunity reactions may be investigated with a view to obtaining information on the following points :

(1) as a means of gauging immunity in an individual on whom the attempt has been made to confer protection by immunisation ;

(2) to prove the occurrence of infection and its nature by demonstrating the presence of antibodies for a known or suspected infective agent in the patient's serum—the “ serodiagnosis ” of disease ; the Wassermann syphilis reaction is analogous, but it is not a true antibody reaction ;

(3) to identify an organism isolated from a patient by proving that it reacts characteristically with an antiserum which has been generated as the result of immunising an animal with a known organism ;

(4) in order to measure the potency of an antiserum with a view to determining its therapeutic value, either as a prophylactic or a curative agent.

The considerations which are advanced here make it clear that these questions can be satisfactorily determined at present only in a relatively small number of instances. The interpretation of immunity reactions in general depends on an intimate appreciation of their scope and of the limitations to which they are subject, and in order that the results of such methods of investigation may be advantageously applied in practice an understanding of what is known as to their nature appears essential

—particular aspects of this problem have already been discussed in connection with the diagnosis of “enterica” (see p. 30).

THE CHEMICAL NATURE OF ANTIBODY REACTIONS

The chemistry of antibody reactions has been actively investigated for upwards of twenty years now, and the results constitute an extensive literature. The stage of simplification has not yet been reached, however, and later work has almost always added to the complexity of the subject. Few doubt the chemical basis of the alterations in the tissues and body fluids which constitute acquired immunity. To speak of immunity reactions, however, may be misleading, and it is more accurate to designate them antibody reactions, since the very opposite of immunity, that is *anaphylaxis*,¹ may result from so-called procedures of immunisation. The manifestations of antibody action are in most cases not peculiar, but consist in physical or chemical alterations which can usually be produced in various other ways. In general, the characteristic feature of an antibody reaction consists in its being brought about by an antiserum, which, apparently, differs from other serum only in one constant feature—namely, its capacity to cause certain changes when

¹ *Anaphylaxis* or *Hypersensitiveness* manifests itself under certain circumstances as a result of the *reintroduction* of an antigen into the tissues and is probably the consequence of an antibody reaction occurring *in vivo* when antibody and antigen react under special conditions as to quantity and tempo (see p. 273).

associated with the substance which stimulated its development in the animal body, *i.e.* the *homologous antigen*. It is extremely difficult to classify antigens except according to the effects which follow when they are associated with the corresponding antibodies. Thus it is possible to differentiate (1) *antitoxic* and *antiferment action*, (2) *agglutination* and *precipitation*, and (3) *complement fixation*, which may or may not be accompanied by *cytotoxic* and *cytolytic action*, (4) *bacteriotropic* or *opsonic effects*; in addition to these there falls to be considered (5) decompositions ascribed to the action of *antagonistic ferments*.

In order to obtain a clearer insight into the processes, it is necessary to consider (*a*) the constitution of antigens and the nature of the changes which they excite in the animal body, thereby leading to the production of antistances, and (*b*) the phenomena presented by the interaction of the constituents concerned in antibody reactions.

THE NATURE OF ANTIGENS

Proteins

An antigen is in general a foreign protein, and it is immaterial whether it is derived from higher animals and plants or from bacteria or protozoa. The two principal features of antigens are: (1) that they are substances foreign to the species of animal which reacts; and (2) that they are usually so intimately associated with protein that it is impossible to obtain antigens altogether free from protein reactions.

The latter characteristic is not shared by all antigens, however, if we are to include those substances which, according to Abderhalden, lead to the development of antagonistic ferments. Thus, Abderhalden found, confirming and extending earlier observations of Weinland, that a comparatively simple substance, such as saccharose, when introduced into the tissues of dogs, might lead to the appearance of invertase in their serum.

With the possible exception of the group of bodies which excite the production of antagonistic ferments and also of Ford's amanita-haemolysin and the toxin of rhus toxicodendron, which he holds to be glucosides, antigenic properties appear to be indissolubly associated with proteins or closely allied substances.

Can Lipoids act as Antigens?

The evidence brought forward to show that any of the so-called lipid bodies, such as "lecithins," in the isolated state can act as antigens is scanty and unsatisfactory.

Jobling and Bull found that a haemolytic immune serum, with or without complement, when brought into contact with the homologous blood corpuscles, caused an increase in acidity, which they attributed to the splitting-off of fatty acids from lipoids contained in the red cells. Non-homologous red cells did not yield so much acid as the homologous corpuscles. It was concluded, however, that the action of the specific immune lipase was not essential for haemolysis, since an immune serum, deprived of its lipase, by heating to 70° C., still produced lysis along with complement. There is no evidence that any immune body is actually a lipid (Meyer). Apart from the singular results of Kurt Meyer obtained with tapeworm lipoids (confirmed by Thiele and Embleton), it has not been found possible

to produce complement-deviating or precipitating antibodies by injecting animals with lipoids (Fitzgerald and Leathes, Ritchie and Miller).

Lipoids are present in a "masked" state in practically all tissues and body fluids, and since it is probably impossible to remove lipid substances completely without profoundly altering the proteins with which they are associated, it is therefore difficult to determine in how far their presence may affect the antigenic action of proteins (see Landsteiner). The weight of evidence, however, goes to prove that where the development of antibodies, in response to lipoids, has been claimed, the possible fallacy due to the entangling ("adsorption") of proteins has not been excluded. As is known from the experiments of Friedberger and Dorner on the development of haemolysins, an extremely minute amount of protein may suffice to lead to the production of recognisable quantities of antistances, and in the work of Bang and Forssman, which originally brought into prominence the question whether lipoids could act as antigens, the immune body developed in response to "lipoid" constituents of red corpuscles was exceedingly feeble.

The Nature of Foreignness

Turning now to the apparently essential element of foreignness in an antigen, it appears that certain infections may so alter the reacting power of the body or may produce such a change in certain tissues that, as a consequence, an individual may develop

antibodies to his own cells. Thus the pathogenesis of paroxysmal haemoglobinuria is to be explained by the fact that certain syphilitics develop a lysin for their own blood corpuscles (see Browning and Watson). It is not known whether this haemolysin is produced as a true antibody response to constituents of the red corpuscles or whether it is of "heterogenetic" origin (see p. 175). The question whether autolysins for other tissues may develop and so lead to disease merits consideration.

THE CONDITIONS OF DEVELOPMENT OF ANTIBODIES BY THE TISSUES

It is not yet possible to arrive at any clear conception with regard to the means whereby antibody production is excited. Recent work on the digestion and resorption of proteins has shown that, in the adult at any rate, the intestinal epithelium rigorously protects against the entrance into the tissues from the alimentary canal of albuminous material with foreign specificity. The direct contact of foreign protein with the tissues, such as occurs in immunisation or in bacterial infections, has therefore no parallel in normal processes of nutrition; accordingly, the reactive phenomena following such "*parenteral*" introduction of protein must be regarded as essentially pathological.

The antigenic power of different proteins varies extraordinarily; thus serum or red blood corpuscles readily produce an antiserum, while tissue

proteins are feeble antigens, so that it is difficult to obtain antibodies to them even after many injections (Salus, Joannovics). Most purified proteins are found to have become seriously impaired as antigens (Wells). Again, certain bacterial products, such as the haemolysin produced by streptococci, would appear to be altogether incapable of giving rise to antistances (McLeod) ; this opens up the possibility that pathogenic products of infection may exist to which the tissues are altogether unable to develop immunity. The question as to what particular character in proteins confers on them antigenic properties is a very subtle one, and obviously is of fundamental importance, but no satisfactory solution has been offered. There is certainly no constant correlation between toxicity and antigenic action,¹ and the term *ictus immunisatorius* conveys little meaning ; except by analogy it is not possible to predict the potency of a given antigen to develop antibodies. This may have an important bearing on the well-known variation in the therapeutic efficacy of vaccines ; these may fail to produce a reaction which is capable of generating antibodies adapted to frustrating the infection.

It is noteworthy that a natural infection may differ markedly from artificial immunisation as a means of stimulating antibody production ; thus the

¹ Griffith, as the result of his investigations on agglutinating antisera for meningococci, has come to the conclusion that increase in virulence is accompanied by increase in complexity of the antigens contained in the organisms.

antibodies developed in the course of an attack of typhoid fever are in general feeble as compared with those resulting from several injections of typhoid vaccine. This may partially explain the efficacy of vaccine treatment in such conditions.

PHENOMENA OF THE INTERACTION OF CONSTITUENTS CONCERNED IN ANTIBODY REACTIONS

Here we are hampered by the fact that antibodies have never been isolated in the pure state, and the same is true of most if not all antigens. Consequently, in an antibody reaction one observes effects brought about by the interaction of certain components in a highly complex and irresolvable mixture. The influence of physical and chemical changes on the course of the reaction can be examined and important results have been obtained, but the question usually remains undetermined as to whether an alteration in the essential components has led to any given change, or whether a modification of the medium is primarily responsible.

Colloids and Physico-Chemical Reactions

Antibody reactions have been extensively explained by reference to physico-chemical and colloid phenomena. The reacting components appear either to be colloids or else to be markedly influenced by close association with such substances. Thus, they tend to be fixed by bodies which act in virtue

of their surface, *i.e.* they are susceptible to adsorption; also, they exhibit frequently the "zone-phenomenon," *i.e.* a particular effect occurs only when the components are present in certain relative amounts, excess of one constituent or the other tending to inhibit the reaction. Adsorption, however, occurs in characteristic form under conditions which exclude the essential feature of antibody reactions, namely, specificity. It has been shown (*vide* von Szily, also Landsteiner and Prasek) that red blood corpuscles treated with osmic acid can remove from an antiserum the immune body which has been developed by injections of red blood cells of a different species, but this does not explain why a certain portion of the immune body can be absorbed by intact corpuscles only when these belong to the homologous species. Again, the fact that passage through a Berkefeld filter removes complement, but not immune body, from the serum (Muir and Browning, see also Schmidt), does not explain why a protein or a suspension of red blood corpuscles should be able to absorb considerable quantities of complement only when the corresponding antibody is present. Alterations in surface tension have been invoked in order to explain immunity reactions (Zangger). The occurrence of this phenomenon, under the name of the *meiostagmin reaction*, has been investigated in connection with the serum of cancer cases, various tissue extracts and lipoids being employed as antigens, but nothing of a conclusive character has been elicited.

Specificity

Specificity, then, is one of the chief problems of antibody reactions. The identification of the species of origin of a twenty-millionth of a gram of serum protein by means of an antibody reaction (complement fixation) is certainly one of the most remarkable of chemical phenomena. Specificity for the species has long been known, but recent observations show that a much more restricted degree of specificity may be exhibited by antibodies. Mackie (see p. 202) found that a powerful antiserum, which agglutinated the culture of *B. coli* by means of which it was developed, in a dilution of 1 in 30,000, was entirely without agglutinating action on numerous strains of organisms derived from a similar source, which appeared identical when tested by all the other available criteria; and further, these strains did not absorb the agglutinins from dilute solutions. On the other hand, complement fixation, brought about under the influence of the same antiserum, demonstrated that in addition to the marked specificity for the homologous strain, there was also a wide group specificity, which included organisms differing markedly in other biological properties such as the power of fermenting lactose. Experiments of this kind show that the determination of biological relationships by means of antisera may depend greatly on the particular test employed. Here also it is impossible to generalise from the behaviour of any one species of antigen, and a law which applies

to the immunity reactions of a particular group of bacteria may be quite inapplicable to others.

According to Ehrlich, the specificity of antibodies is a result of differences in chemical structure of the antigens in response to which they have been developed. This view is strikingly borne out by the work of Obermeyer and Pick, and of Landsteiner and his co-workers, who have shown that specificity can be modified by chemical procedures. Thus they have distinguished *structural* from *species specificity*. Proteins altered by physical processes, such as boiling, give rise to antibodies which react only with boiled protein; the latter, however, must belong to the species employed for immunisation, that is, the species specificity is preserved. On the other hand, Landsteiner and Jablons have found that rabbits injected with acetylated horse serum develop antibodies which fix complement in the presence of acetylated serum protein from various other animals (hen, rabbit) in addition to the horse, but do not absorb complement with normal or boiled horse serum or with diazo- or nitro-serum protein. Rabbits will also produce an antiserum to acetylated rabbit serum. Accordingly, the process of acetylation has deprived the serum of those characters which distinguished it as belonging to the species horse, and has conferred on it a form of specificity which depends on the presence of the acetyl group (structure specificity). In respect of specificity, it is significant that a clear distinction exists between an animal's tissues and its serum (Fleischmann and

Davidsohn), since antibodies obtained by injecting various organs do not fix complement in the presence of serum from the same species. An antiserum to globin, the protein constituent of haemoglobin, does not fix complement along with serum, intact red corpuscles, haemoglobin, or acid serum-albumin (Browning and Wilson).

Natural and Heterogenetic Antibodies

While the specificity of antisubstances developed by immunisation is so striking and constant, it must be remembered that similar highly specific bodies occur in serum altogether apart from reaction to the introduction of an antigen: thus a lytic antibody for ox blood is present frequently in the serum of normal guinea-pigs. Accordingly, *the development of an antibody need not necessarily possess any teleological or etiological significance.* This consideration serves at least to rob of its apparently illogical character the formation of "heterogenetic" antibodies (Friedberger), as when a rabbit injected with guinea-pig's kidney develops an immune body for sheep's red blood corpuscles (Forssman). Thus, the introduction of certain foreign proteins into the tissues causes a metabolic change one of the constant manifestations of which is the development of this particular immune body. A case in point is the development of a positive Widal reaction for *B. typhosus* in the serum of cases of typhus fever (Wilson); Ballner and Sagasser discuss heterogenetic agglutinins extensively. The capacity to produce a

positive Wassermann reaction is probably a somewhat similar heterogenetic phenomenon; thus, a property which is common to the serum of many normal rabbits (Browning and Mackenzie) appears in human serum practically only as the result of syphilitic infection. Such a constant phenomenon possesses great diagnostic value, but is probably to be regarded merely as the indicator of pathological processes.

SPECIAL FEATURES OF CERTAIN ANTIBODY REACTIONS ¹

Antitoxins and Antiferment Action

In *antitoxin reactions* the important fact has been recently elicited that the toxin is not destroyed; on the contrary the combination remains permanently reversible provided that a dissociating factor is introduced, such as acid (Morgenroth and Asher) or alkali (Sachs).

Fresh normal serum inhibits the action of trypsin; this *antitryptic* property—the true nature of which is obscure (see Wells for literature)—has been held to explain in part the mechanism of defence against infection in wounds, since normal fresh serum is a less suitable medium for the growth of some organisms than is serum whose antitryptic action has been neutralised by adding to it some source of trypsin, *e.g.* dead leucocytes.

¹ The general features of such reactions are dealt with in the author's article on Immunity in Pembrey and Ritchie's *General Pathology*, London, 1912.

Agglutination and Precipitation

The occurrence of zones, which is so characteristic of colloid interactions, is strikingly shown in this group of reactions. Dreyer and Jex-Blake have proved that the explanation of "zones" (see p. 172) as due to "proagglutinoids" does not apply generally. It had been supposed that the agglutinin, as the result of heating, became converted into proagglutinoïd, which possessed in marked degree the power of combining with the bacteria, but which had lost the property of causing agglutination. A suitably heated serum, however, may actually exhibit increased agglutinating power and yet produce a marked zone, whereas a serum much weakened by heat may cause no zone. Further, zones may be produced by employing heated bacteria instead of heated serum.

The analysis of the mechanism of agglutination reactions is complicated by the fact that the visible end-result, agglutination, represents not merely the effect of a specific combination of the antibody with the bacteria, but also requires for its occurrence the co-operation of a suitable medium. Bordet showed that, in the absence of a salt (or sugar), as when the bacilli are suspended in distilled water, no agglutination occurs, even although the agglutinin is bound; accordingly the *loss of agglutinin by absorption from a serum is the true criterion of reaction* (see p. 28).

In certain instances agglutination depends on the

action of complement as well as of antibody (Muir and Browning), and Bordet and Gay have shown that thermostable constituents of ox serum (conglutinin) play a peculiar part in causing agglutination in the presence of antibody and complement.

Complement Fixation, Cytolysis, and Cytotoxic Action

Certain antibodies, when associated with the corresponding antigens, deprive fresh serum of its complement. The fixation of complement has been explained as due to adsorption. But the mere occurrence of a serum precipitate is not sufficient to fix complement; thus, globin when mixed with any serum gives rise to a precipitate, but it is only when an antiglobin immune serum is employed that complement is bound (Browning and Wilson). Dean has shown that the amount of complement absorbed by a serum *plus* antiserum complex may depend to a marked extent on physical conditions, and varies according as the components are mixed simultaneously or the complement is added after the mixture of antigen with antibody has been allowed to stand for some time. The degree and rate of fixation may be influenced also by temperature (see Dean) and by reaction (Browning and Wilson). It is, further, a noteworthy fact that with certain antigens, for example, serum, zones occur, so that if an excess of antigen is present complement fixation fails to take place; this is obviously of great practical importance. Comple-

ment fixation may occur without visible formation of precipitate; and Arrhenius cautions against the assumption that adsorption takes place in such instances where there is not definitely a solid phase present. Bechhold's observation that colloidal arsenic trisulphide ceases to be filtrable when mixed with a colloidal solution of Berlin blue, although the mixture is quite transparent, suggests, however, that colloidal solutions may act as adsorbing agents.

It is to be noted that the occurrence of adsorption phenomena may depend intimately on the chemical structure of the adsorbing agent, and a slight alteration in constitution may alter markedly the adsorptive property. For example, derivatives of cholesterin which fix lecithin, as tested by inhibition of snake venom lecithin haemolysis, do not differ from those which lack this power so far as can be measured by a physical character, such as the rapidity of precipitation out of emulsions with lecithin (Browning and Cruickshank).

Lytic and Lethal Action on Cells, Bacteria, etc.—Cytolysis may or may not follow complement fixation; but, so far as we know, suitable complement is essential for the occurrence of this group of effects. A large amount of complement may be absorbed by the combination cell-receptor *plus* antibody, depending on the character and quantity of the immune body present as well as on properties of the complement peculiar to the species or the individual. For the production of lysis a certain minimum amount both of immune body and of complement is

required, hence they do not behave precisely as ferments; and there is also no satisfactory ground for regarding the using up of complement as a mere side-phenomenon ("methaemolysis"—see Bordet). As regards the part played by the immune body, Muir long ago observed that on increasing the amount of this component beyond the minimum quantity necessary to cause haemolysis, an increase was also in many instances produced in the amount of complement fixed by the immune body-receptor complex. It has been shown by Browning and Wilson, confirmed by Thorsch, that this property of excess of immune body to lead to increased complement fixation depends to a great extent on the stage of immunisation at which the antiserum is taken; thus, an immune body obtained from the rabbit after a single injection of washed ox blood has, when multiple doses are added to the corpuscles, only a feeble power of causing increased fixation of complement, whereas the "mature" immune body behaves as Muir has described. Thiele and Embleton have described further striking differences between mature and immature lysins. The whole question of differences between what we have termed "mature" and "immature" antibodies is one of great importance in relation to the processes of natural disease; it must be borne in mind that most experimental work has been done in connection with the phenomena in very highly immunised animals, whereas recovery from a naturally acquired disease is in general not accompanied by

enormous production of antibodies. It is probable that the basis of immunity in spontaneous recovery is widely different from that in artificial immunisation; but the investigation of low grades of immunity presents very great difficulties.

It is an important fact that the lytic power of a complement depends on the species of animal yielding the immune body through whose medium it is brought into combination with the red corpuscles (Muir); and this holds even when the immune bodies appear to combine with the same receptors of the red cells. In view especially of Muir's work on the subject there is now no reason for applying the misnomer "amboceptor" to immune body.

The capacity for being fixed by antigen *plus* antibody, i.e. *deviability*, of the complement of one individual may differ markedly from that of another member of the same species, and this property is quite independent of the lytic power, as Browning and Mackenzie pointed out. *On what function differences in deviability depend has not yet been ascertained, but it is a point of the greatest practical importance in the interpretation of the results of complement-fixation experiments, e.g. in the Wassermann reaction, and one which has not hitherto been sufficiently well recognised.* Browning and Mackie have drawn attention to the value of the well-known method of freezing as a means of preserving a standard complement with constant deviability and lytic action over a considerable period. By procedures such as dialysis it is possible to reduce

the deviability of complement greatly without effecting practically any alteration in its haemolytic power (Browning and Mackie).

Constitution of Complement.—The complementing function of a serum is a complicated property and extremely labile, but it withstands the action of ammonium sulphate, and by making use of this reagent, in addition to Liefmann's carbonic acid method, for the purpose of fractionation much more uniform and satisfactory analyses have been obtained than from the carbon dioxide procedure by itself. Of course, it is not assumed that the various fractions so obtained are homogeneous protein entities, but the fact is to be emphasised that it is possible with great regularity to split complement up into four different components, and that certain combinations, consisting as a rule of at least three of these, quantitatively reproduce the full haemolytic effect of the native serum (Browning and Mackie). This process of reconstitution is not a mere summation of effects, and the fractions do not correspond with the hitherto described end-piece, middle-piece, and third component. In general, the major portion of complement is represented by the following combinations of two constituents: (1) the pseudo-globulin components of each carbon dioxide fraction together, and (2) the pseudo-globulin of middle-piece along with the serum-albumin. Frequently, however, the addition of the albumin to the mixture of globulins is required in order to reconstitute the complement, so that the co-operation of three components may

be necessary. Thus, while the complement factors are distributed over the pseudo-globulins principally and may be wholly contained in these, the albumin also in many instances constitutes an essential element. Euglobulin *plus* albumin often exert marked complementing action, but the addition of one of the pseudo-globulin fractions is required to restore the full power. The terms mid-piece and end-piece are so indefinite in their application that it would be better to give them up altogether.

Attempts have been made to relate the inactivation of complement brought about by heat and other agencies to alterations in surface tension, but Schmidt has shown that no inference can be drawn as to the complement action of a serum from its surface tension.

In spite of much effort (v. Liebermann and v. Fennyvessy) no true substitute has been found for complement in the haemolytic system. On the other hand, it is a fact of great theoretical importance that immune body can be replaced by colloidal silicic acid (Landsteiner and Jagic). It may be taken as definitely established that the component of serum which acts in this case is identical with the complement which causes lysis along with immune body (see Browning and Mackie); but the specificity which characterises immune body is wanting, as silicic acid sensitises various species of red blood corpuscles to the action of complement. The alteration produced in the red corpuscles by immune body is no doubt analogous to that effected

by silicic acid, but this does not elucidate the nature of the change, nor does the observation of v. Liebermann and v. Fennyvessy that corpuscles sensitised by immune serum are rendered more susceptible to the lytic action of soaps, explain the process of serum haemolysis. Certain lipoids—"lecithin"—may markedly modify complement action (Cruickshank and Mackie), but there is no evidence that lipoids can replace either immune body or complement in the haemolytic system. Chick has found that when pseudo-globulin of antidiphtheria serum is dialysed for a long time denaturation occurs, resulting in a precipitate of euglobulin, which ceases to form when most of the phosphorus (contained in "lecithin") has been removed from the soluble components, the latter retaining all the antitoxin. This observation is of great interest as regards the rôle of lipoids in relation to antibodies.

The difficulty which may attach to the analysis of haemolytic processes is well shown by haemolysis due to brilliant green along with serum (Browning and Mackie). Thus ox blood treated with brilliant green is lysed by minute amounts of serum (0.005 c.cm. serum for 1 c.cm. 5 per cent. washed blood suspension) belonging to various species (human, guinea-pig, ox, etc.); but this result does not depend on complement, as Mackie has shown that serum diluted with ten volumes of physiological NaCl solution and then boiled still acts. The following is a summary of unpublished observations of Mackie on these points: The optimum effect is

produced when the dye is allowed to act on the red corpuscles, and the latter are then well washed by centrifugalising with 0·85 per cent. NaCl solution. Lecithin also lyses red corpuscles sensitised in this way. But the addition of small amounts of the dye to the lecithin emulsion or to serum deprives these agents of their "activating" effect. Egg-white does not lyse corpuscles treated with brilliant green ; but serum precipitated with alcohol and repeatedly treated with alcohol and ether still acts. The amount of brilliant green which causes the sensitising action is a minute fraction of the quantity which by itself causes lysis. At present it is not possible to give any explanation of the phenomenon, but it affords a striking example of the subtlety of haemolytic reactions.

The Wassermann syphilis reaction probably belongs to the group of heterogenetic phenomena (see p. 175). No other non-specific alteration of serum, in the etiological sense of the term "specific," has been found to be so constantly present in any disease or so completely restricted to one particular condition. But the nature of the interaction between the constituents of a positive serum and the lipoid "antigen" remains quite obscure in spite of many ingenious efforts at explanation. Our only means of estimating it is through the occurrence of inhibition of that equally complex function complement. It is to be noted that there is no proof that a positive and negative serum are qualitatively different ; the difference is quantitative.

The essence of the syphilitic "antigen," whether it be an emulsion of so-called lecithin or of a mixture of lecithin with cholesterin, is that in the presence of syphilitic serum it leads to the fixation of more complement than is fixed under similar conditions in the presence of normal serum ; to speak of a pure syphilitic antigen conveys no precise meaning at present, since no chemical entity of the nature of "lecithin" has so far been prepared with certainty. Cholesterin, when added to the "lecithin" antigen, has the remarkable property of increasing the amount of complement fixed in the Wassermann reaction, as was first shown by Browning, Cruickshank, and Mackenzie. Under suitable conditions this effect is strictly specific, and cholesterin causes no increase in the anticomplement action of the antigen alone or together with a non-syphilitic serum. It has been found that the properties of alcoholic tissue extracts as syphilitic antigens are in general enhanced by the addition of cholesterin, and our observation has now met with an almost universal application in the practice of the Wassermann reaction.

Wassermann and Lange have attempted to obtain an insight into the changes which lead to a positive reaction in the following fashion: a positively reacting cerebro-spinal fluid rich in lymphocytes was centrifugalised so as to concentrate the cells ; a small specimen of the cell-free fluid, and a similar specimen to which the concentrated cell content was added, were then heated for a prolonged period at 57° C. It was found that, as a result of this procedure, the specimen containing the cells reacted more markedly positive than the cell-free portion. Negatively reacting fluids containing cells, from cases of menin-

gitis, were not rendered positive by a similar procedure. The conclusion was that the reacting bodies may reside within the lymphocytes in syphilis. Although by no means convincing, these suggestive experiments are well worth further analysis.

Bacteriotropic and Opsonic Action

The important observations of Wright and Douglas aided greatly in demonstrating the complementary characters of the humoral and the cellular aspects of immunity phenomena, since they showed that under certain circumstances bacteria were not ingested by leucocytes until the former had been acted on by constituents of the serum, which were accordingly termed "opsonins." Muir and Martin then effected an advance of the greatest significance by proving that normal opsonins possessed the characters of complement, whereas immune opsonins were true antibodies. Browning found also that opsonic effects in certain instances depended on the co-operation of complement with antibody, each by itself being practically devoid of opsonic action.

It is clear that for the production of immunity phagocytosis is only one step in the process. Unless the ingested organisms are subsequently inhibited in their activity or destroyed, little benefit will accrue to the infected individual. But the factors on which the occurrence of this second stage within the cell depends are as yet unknown.

Antagonistic Ferments (Abderhalden's Reaction)

Abderhalden's conception of antagonistic ferments proceeded from the fact that foodstuffs, especially

protein, undergo a marked degree of splitting up in the intestine before they are absorbed into the tissues and there synthetised so as to become genuine constituents of the body. The reason for this process is supposed to be that the protein taken as food is foreign—that is, does not contain the proper proportion or association of the constituents required for building up the animal's own tissues; accordingly the foreign protein is broken down to such an extent as will enable the intestinal cells to pick and choose the proper components in suitable amounts. On this basis Abderhalden has concluded that when a foreign substance, *e.g.* protein or carbohydrate, is introduced into the tissues parenterally—that is, otherwise than by the alimentary canal—the result is the development in the body-fluids of antagonistic ferments which break down the substance in a fashion similar to that which occurs in the process of digestion in the alimentary canal; in addition, the antagonistic ferment thus produced is supposed to be specific for the substrate in response to which it has been developed. Thus, in pregnancy the foetal elements of the placenta obtain access to the maternal circulation, and in virtue of their hypothetically foreign character they are supposed to lead to the production of ferments which digest placental tissue. For the detection of the products of ferment action two methods have been employed—(1) the altered rotation of the plane of polarisation, resulting when the serum acts on a solution of peptone derived from the tissue in question,

and (2) the demonstration of diffusible products—amino-acids—which pass into the external fluid when the serum and boiled substrate are allowed to act on each other in a dialyser; for this purpose Ruhemann's reagent triketo-hydrinden-hydrate, "ninhydrin," has been mainly employed.

The differences in rotation observed by the optical method are generally small. With regard to the ninhydrin reaction, unfortunately a sharp line of demarcation cannot be drawn between the violet colour which is interpreted as a positive result and a complete absence of colour; an intermediate series of yellowish and reddish tints occurs, the true value of which it is extremely difficult to assess. Also, it is difficult to obtain dialysers of constant permeability for the substances under consideration.

The cause of the Abderhalden phenomenon is not yet clear, and the gaps in knowledge regarding it are wide.

Observations by J. M'Vey in the Pathological Department of Glasgow University, with a view to correlating the phenomena with other immunity reactions, led to the following results:

(1) The reaction is a quantitative one; serum along with various proteins will produce diffusible substances reacting to ninhydrin, provided that sufficient serum is employed—for example, normal rabbit's serum acting on egg-white; (2) the reaction is diminished or abolished if the serum is heated sufficiently beforehand (60° C. for an hour); (3)

the injection of an immunising dose of egg-white (5 c.cm. 20 per cent. solution) intravenously into rabbits causes little or no alteration in the power of their serum to give a positively reacting dialysate when tested with egg-white seven to ten days later ; (4) the lysis of washed ox blood by the fresh serum of an immunised rabbit gives rise to only a slight degree of proteolysis, as evidenced by the ninhydrin reaction when the mixture is dialysed in the usual fashion ; fresh normal rabbit's serum also gives rise to diffusible ninhydrin reacting substances when placed in contact with ox's blood corpuscles, although practically no haemolysis occurs in this case ; heated immune rabbit's serum (one hour at 55° C.) along with ox's corpuscles does not yield a positively reacting dialysate. Normal guinea-pig's fresh serum, although it contains much more complement than rabbit's serum, does not necessarily cause a positive ninhydrin reaction when it produces lysis of ox blood sensitised with multiple doses of immune body from the rabbit. Accordingly, there would not appear to be any constant correlation between the Abderhalden reaction and other antibody reactions.

As the results of many observers indicate, the diffusible bodies which react with ninhydrin are produced more readily in the presence of coagulated placenta by the serum in pregnancy than by the sera of normal individuals ; but this is not an invariable rule, and also certain specimens of placental tissue yield a positive ninhydrin reaction

with various non-pregnant sera. De Waele has come to the conclusion that the bodies which react with ninhydrin are derived from the serum and not from the substrate, while independently both Leitch and Plaut have shown that serum on incubation with inorganic substances, such as kaolin or glass wool, without any organic substrate, may give rise to positively reacting dialysates; further, a given serum may develop the reaction in the presence of one of these inorganic bodies, and not with another. In view of these results, the specific nature of the reaction for antagonistic proteolytic ferments appeared extremely doubtful, and this conclusion may be taken as completely substantiated by the further work of van Slyke, Vinograd-Villehur, and Losee. They determined the amino-nitrogen after the mixture of serum and substrate had been incubated and then protein had been removed by colloidal ferric hydrate; they found that this procedure yielded values of amino-nitrogen many times greater than the experimental error when digestion occurred. By this method they ascertained that "the individual variations of both pregnant and non-pregnant sera make the results from both overlap so completely as to render the reaction, even with quantitative technique, absolutely indecisive for either positive or negative diagnosis of pregnancy"; and again, "further evidence of non-specificity is seen in the fact that carcinoma tissue was digested to about the same extent as was placenta."

The idea that in disease the body might produce antagonistic ferments to certain of its own organs opened out a wide prospect to investigators, and numerous remarkable results have been recorded ; but granting even that the reaction possessed a certain degree of constancy as a phenomenon of pregnancy, the above-mentioned facts rendered it highly inadvisable to conclude that a particular organ was affected solely on the ground that the patient's serum, along with that tissue as substrate, gave a positive Abderhalden reaction. *It is now practically certain that this phenomenon is devoid of value in the diagnosis of pregnancy or disease.*

*Is there a Multiplicity of Antibodies generated
by a Single Antigen ?*

The question whether the various antibody reactions which may result from immunisation with a given substance are really due to separate antibodies is important ; the evidence goes to prove that a considerable degree of independence exists. Thus Muir and Martin have shown, in the case of bactericidal, opsonic, and agglutinating properties of immune sera, that these functions vary independently ; further, Mackie's observations on agglutination and complement fixation in the *B. coli* group, point in the direction of independence of the antisubstances, as do also Hektoen's experiments in which dogs injected intravenously with rats' red corpuscles developed agglutinin and immune opsonin in their

blood serum, while the cerebro-spinal fluid contained immune opsonin only.

On the other hand, a given species of organism may contain several different antigens; if the relative proportion of these antigens varies in different strains of the organism, then antisera generated by each will differ and the antibody reaction will be most marked when the corresponding antigen and antibody meet. This would explain the occurrence of several different serological types in one species of bacteria, *e.g.* pneumococci (Cole) or meningococci (see Gordon, also Griffith). In the case of bacilli of the coli group an even greater multiplicity of antigens must be assumed (see p. 202).

THE SCOPE OF MATHEMATICAL METHODS

The analysis of antibody reactions by mathematical methods (Arrhenius, McKendrick, and others) has led to results which, if they could be accepted as general laws, would effect a most acceptable simplification. From the biological aspect, however, such generalisations cannot be regarded as universally applicable in the meantime. There are several reasons for this: (1) The active substances which are being measured—for example, in serum—are modified by the presence of a large quantity of matter which is prone to exert more or less unspecific effects. Thus the action of complement is retarded or inhibited altogether when it is mixed with a specimen of the same serum which has been

heated (Muir and Browning), yet it is certain that the complement is not destroyed in the mixture, but merely masked—in this instance complementoid was shown to be largely responsible for the inhibition.

(2) Individual variations constitute an exceedingly well marked feature of immunity reactions. The independence of deviability and haemolytic power of complement in the serum of different individuals of the same species illustrates this point well. Another striking example is the difference in affinity of antibodies for the respective antigens. Thus the immune body obtained from rabbits injected with ox blood has a high degree of affinity for the red cells, so that the treatment of the antiserum with the blood corpuscles removes multiple doses of the immune body; on the other hand, the normal antibody for guinea-pig's blood contained in ox serum has by itself little or no affinity for the red cells.

These variations among bodies closely similar in biological action, as well as the multitude of interfering conditions, seem to present an almost insuperable obstacle to the elaboration of general formulae. In view of the high complexity of the substances which take part in antibody reactions it is difficult to escape the conclusion that such a statement as that any given effect, *e.g.* bactericidal action, occurs in accordance with the law of a monomolecular reaction, is merely the expression of a superficial similarity, which may tell one really nothing of the underlying processes. Thus it is likely that the most which mathematical analysis

can hope to accomplish for a long time to come in the majority of such biological problems is merely to afford a graphic representation of ascertained facts. The discovery or prediction of "laws," which is so valuable a result of mathematical methods as applied to phenomena of pure physics, is probably out of the question here.

On the other hand, *statistical methods* afford indispensable assistance in checking conclusions arrived at on an insufficient numerical basis. Thus it has been shown that the "opsonic index," as originally calculated, is practically devoid of value, clinically, since the number of organisms ingested by fifty or a hundred leucocytes is affected by "random sampling" to so great a degree that variations in the result, such as the authors of the method considered to be significant, are almost a matter of mere chance (Fitzgerald, Whiteman, Strangeways, Greenwood and White).

SUMMARY OF PRACTICAL APPLICATIONS OF ANTIBODY REACTIONS

(*In this list are included the applications whose value and reliability are generally recognised; less assured examples are placed within brackets.*)

ANTITOXIC ACTION

Recognition of Antibody.—(1) For the estimation of the therapeutic potency of antisera, *e.g.* antidiphtheria, antitetanus, and antiscorpion sera. As a rule what is actually determined is the power of the antiserum to neutralise toxin when the two are mixed *in vitro*; the presence of unneutralised toxin

is then tested for by injecting the mixture into a susceptible animal. In the case of antisera employed for therapeutic purposes, *e.g.* diphtheria antitoxin, there is much evidence in favour of the view that the antibody content of the serum, as estimated in this fashion, is an accurate index of its curative power, although this is not universally accepted.

(2) In order to gauge an individual's immunity to diphtheria. A minute dose of toxin is injected subcutaneously; the development of a local reaction—*Schick's reaction*—shows deficiency of immunity and hence danger of contracting the disease on exposure to infection with *B. diphtheriae* and is thus taken as evidence of the need for immunisation.

Recognition of Antigen.—An organism can be identified as true *B. diphtheriae* or *B. tetani* if its pathogenic action is annulled on inoculating a susceptible animal with a culture to which has been added antitoxin produced by immunisation with a known strain of the organism in question.

AGGLUTINATION

Recognition of Antigen—for the identification of certain pathogenic organisms, isolated from the tissues and excretions of patients or other sources, especially the typhoid-paratyphoid, dysentery, and cholera groups—a practically certain method in the case of these organisms (see p. 22). In the case of pneumococci and meningococci it is found that several distinct *types* exist, each of which is agglutinated powerfully by an antiserum generated by

a member of its type, but is only slightly affected by antisera to the other types.

Recognition of Antibody—with patients' sera the "Widal reaction" is useful for the detection of typhoid-paratyphoid (see p. 30), and also *M. melitensis* infections. It must be noted that the evidence is merely presumptive. (Under the name of the *Weil-Felix reaction*, the agglutination of a bacillus of proteus type by the serum of typhus fever cases has been found of great value in diagnosis—see *British Medical Journal*, vol. i. 1918, p. 65.)

PRECIPITATION

Recognition of Antigen.—(1) The detection of the species-origin of serum protein—less delicate than complement fixation.

(2) The diagnosis of anthrax infection by demonstration of the antigen in decoctions of the tissues of infected animals—*Ascoli's thermo-precipitin reaction*.

COMPLEMENT FIXATION—BORDET-GENGOU REACTION

Recognition of Antigen.—Invaluable for the identification of the species-origin of serum proteins, *e.g.* in blood-stains; similarly for the detection of adulteration in flesh foods, *e.g.* with horse flesh. For identification of certain organisms and their detection in mixtures with others, *e.g.* *B. typhosus* in a mixed culture with coliform bacilli. (As a means of determining the antigenic value of a vaccine with a view to estimating its capacity to produce

immunity in the human subject and hence its power to protect against infection or to contribute toward cure.)

Recognition of Antibody—in patient's sera, the *Wassermann Syphilis test*—not a true antigen-antibody reaction. (The diagnosis of chronic gonococcal infection.)

(As a means of measuring the therapeutic value of certain antisera ; this is a very doubtful application.)

BACTERIOLYSIS AND BACTERICIDAL ACTION

Recognition of Antigen.—A vibrio is identified as true *V. cholerae* if when injected into the peritoneum of a guinea-pig along with true anticholera serum bacteriolysis occurs—*Pfeiffer's reaction*.

Recognition of Antibody.—(The attempt is made to measure the potency of certain antisera, *e.g.* for pneumococci and streptococci, by testing the effect of mixtures of antiserum and virulent organisms when inoculated into susceptible animals.)

REACTION FOR HYPERSENSITIVENESS—ANAPHYLAXIS

(1) For the recognition of infection, especially tuberculosis and glanders in animals (also, *Noguchi's luetin reaction* in latent syphilis).

(2) As a preliminary to serum therapy in order to determine the tendency to serum disease (see p. 274).

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XI

THE IMMUNITY REACTIONS OF THE COLI GROUP

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Mackie, *Journ. of Path. and Bact.*, vol. xviii, 1913, p. 137.

AGGLUTINATION

THE immunity reactions of bacilli belonging to the coli group exhibit a striking contrast to the action of immune sera on related organisms, such as *B. typhosus*, *B. paratyphosus*, etc. Thus, an immune serum to a particular strain of *B. typhosus* will agglutinate the great majority of other typhoid strains with comparatively small variations in degree. Immune sera to various *B. coli* types, on the other hand, have been found to exert little or no action on other strains which correspond in fermentative reactions and other criteria (that is, presence or absence of motility, indol formation) to that used for immunisation. This high degree of specificity on the part of *B. coli* agglutinins towards particular strains was originally observed at a time before

it was definitely realised that it was possible by means of fermentative reactions to separate coliform bacilli into a large number of different varieties ; but even after a series of strains which correspond in fermentative and other reactions has been selected, one is still unable to find a corresponding behaviour with an agglutinating antiserum (Tables I and II).

Agglutinating sera were obtained to strains having the following characters :

Organism.	Motility.	Indol.	Glucose.	Lactose.	Dulcitate.	Saccharose.	Adonite.	Inulin.	Inosite.	Salicin.	Raffinose.	Dextrin.	Glycerin.	Voges and Proskauer Reaction.
I	+	+	+	+	+	+	—	—	—	+	+	—	+	—
II	+	+	+	+	—	—	—	—	—	+	—	—	+	—
III	—	+	+	+	—	—	—	—	—	—	—	—	+	—

I corresponds to MacConkey's bacillus No. 71 ; II, to B. Grünthal ; III, to B. vesiculosus ; and a number of strains, corresponding in all the reactions above noted with these three organisms, were tested with the particular agglutinating sera. The only strains agglutinated to any significant degree were those used for immunisation (*vide* Tables I and II).

In fact, *the action of a coli agglutinin is practically limited to the particular strain used for immunisation*

Paragglutination

While this specificity of agglutinins for particular strains is the general rule in the coli group, an exception has been met with ; thus, an antiserum to a B. coli communis (Escherich) type (Strain IV) was found to agglutinate a particular bacillus,

TABLE I—AGGLUTINATION

ANTISERUM TO STRAIN I (NO. 71 MACCONKEY)

Dilution	1 : 100	1 : 500	1 : 1000	1 : 5000	1:10,000	1:20,000	1:30,000	1:50,000
Strain I	++++	++++	++++	++++	++++	++++	+++	+

OTHER STRAINS HAVING SAME CHARACTERS

Strain <i>a</i>	++	+	0	0	0	0	0	0
" <i>b</i>	+	0	0	0	0	0	0	0
" <i>c</i>	0	0	0	0	0	0	0	0
" <i>d</i>	+++	++	0	0	0	0	0	0
" <i>e</i>	0	0	0	0	0	0	0	0
" <i>f</i>	0	0	0	0	0	0	0	0
" <i>g</i>	++	+	0	0	0	0	0	0
" <i>h</i>	+	0	0	0	0	0	0	0
" <i>i</i>	0	0	0	0	0	0	0	0
" <i>j</i>	0	0	0	0	0	0	0	0

Controls without antiserum showed no agglutination.

TABLE II—AGGLUTINATION

ANTISERUM TO STRAIN II (B. GRÜNTAL)

Dilution	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 8000	1:10,000
Strain II	++++	++++	++++	++++	++++	++++	++++	+++	+
Other Corre- sponding Strains	<i>k</i>	+	0	0	0	0	0	0	0
	<i>l</i>	0	0	0	0	0	0	0	0
	<i>m</i>	0	0	0	0	0	0	0	0
	<i>n</i>	+	0	0	0	0	0	0	0
	<i>o</i>	0	0	0	0	0	0	0	0
	<i>p</i>	+	0	0	0	0	0	0	0
Strain I (ba- cillus No. 71)	<i>q</i>	0	0	0	0	0	0	0	0
		+++	+	0	0	0	0	0	0

ANTISERUM TO STRAIN III (B. VESICULOSUS)

Dilution	1 : 60	1 : 120	1 : 240	1 : 480	1 : 960	1 : 2000
Strain III	++++	++++	++++	+++	+++	++
Other Corre- sponding Strains	<i>r</i>	++	+	0	0	0
	<i>s</i>	+	0	0	0	0
	<i>t</i>	0	0	0	0	0
	<i>u</i>	0	0	0	0	0
	<i>v</i>	0	0	0	0	0
Strain I (bacillus No. 71)	+	0	0	0	0	0

Controls without antiserum showed no agglutination.

No. 71 (MacConkey—Strain I), in a four times weaker concentration than the homologous strain. A second antiserum to the same strain (IV) behaved in practically the same way, indicating that this property of paragglutination was a function of this particular strain. The bacillus No. 71 strain (I) was found to be practically insusceptible to other coli agglutinins (*vide* Tables II and III).

TABLE III—AGGLUTINATION

FIRST ANTISERUM TO STRAIN IV (B. COLI COMMUNIS—ESCHERICH)

Dilution	1 : 100	1 : 1000	1 : 8000	1 : 10,000	1 : 15,000	1 : 20,000	1 : 30,000	1 : 40,000
Strain IV	++++	++++	++	+	0	0	0	0
Strain I = Bacillus No. 71 (MacConkey)	++++	+++	+++	+++	+++	+++	++	++

Ten other Strains having same characters as Strain I showed no agglutination with antiserum diluted 1 : 500.

SECOND ANTISERUM TO STRAIN IV

Dilution	1 : 200	1 : 400	1 : 1000	1 : 2000	1 : 4000	1 : 8000	1 : 16,000
Strain IV	++++	+++	+++	++	0	0	0
„ I	++++	+++	+++	+++	++	+	0

Controls without antiserum showed no agglutination.

Anti-Paracolon Sera

In contrast to these experiments are the results obtained with antisera to two types of paracolon bacilli from cases of pyuria and bacilluria (see table, p. 16). Their cultural characters were as follows :

Organism.	Motility.	Glucose.	Lactose.	Dulcitol.	Saccharose.	Mannitol.	Indol.
A . . .	—	+	—	—	—	+	—
B . . .	+	+	—	—	—	+	—

It was found that other strains similar to the strain used for immunisation were agglutinated to a corresponding degree by the antiserum. This is similar to what is found in the case of an antityphoid serum.

COMPLEMENT FIXATION

The same degree of specificity for individual strains that exists in relation to agglutination also holds in complement fixation experiments¹; but of even more striking interest is the group reaction elicited with these antisera (Table IV). In the case of the Grünthal (Strain II) antiserum, already mentioned, over fifteen doses of complement were absorbed by the combination of 0.001 c.cm. of antiserum and the homologous bacillus, while in the case of another bacillus of the same type (Strain C) it was found necessary to employ 0.025 c.cm. of antiserum to obtain this amount of complement fixation, and on diminishing the amount of antiserum there was a rapid falling off in the amount

¹ Bacterial suspensions were employed similar to those used for sedimentation tests (see p. 25), care being taken to avoid admixture with particles of agar. The mixtures of bacteria, antiserum and guinea-pig's complement were incubated for 1½ hours at 37° C., then the hæmolytic system (Ox RCs + 5 doses of IB from the rabbit) was added and the tubes were again placed at 37° C. for 1 hour.

of complement fixed (from no lysis with twenty doses with 0.025 c.cm. antiserum to almost complete lysis with ten doses with 0.01 and with two doses with 0.001 c.cm.). With representatives of other coli types commonly met with, although they differed very considerably in their characters from the homologous strain, the amounts of complement fixed

TABLE IV—COMPLEMENT FIXATION

Antiserum to Strain II (B. Grünthal).	Bacillary Emulsion.	Doses of Complement.					Complement fixed by Emulsion alone.	
		2 D.	5 D.	10 D.	15 D.	20 D.	2 D.	5 D.
0.025 c.c.	0.4 c.c. Strain II (B. Grünthal)	0	0	0	0	0	Trace	Just complete
0.01 c.c.		0	0	0	0	0	—	—
0.005 c.c.		0	0	0	0	0	—	—
0.001 c.c.		0	0	0	0	Complete	—	—
0.0005 c.c.		0	0	Almost complete	Complete	—	—	—
0.0001 c.c.		0	Distinct	Complete	—	—	—	—
0.025 c.c.	0.4 c.c. Strain C (Non- homolo- gous B. Grünthal)	0	0	0	0	0	Just complete	Complete
0.01 c.c.		Distinct	Marked	Almost complete	Complete	—	—	—
0.005 c.c.		Marked	Almost complete	Complete	—	—	—	—
0.001 c.c.		Almost complete	Complete	—	—	—	—	—
0.025 c.c.	0.4 c.c. Strain D (B. vesicu- losus)	0	0	0	0	0	Very marked	Complete
0.01 c.c.		Distinct	Distinct	Marked	Complete	—	—	—
0.005 c.c.		Distinct	Marked	Complete	—	—	—	—
0.001 c.c.		Marked	Just complete	—	—	—	—	—
0.025 c.c.	0.4 c.c. Strain E (bacil- lus No. 71)	0	0	0	0	0	Marked	Almost complete
0.01 c.c.		0	Distinct trace	Very marked	Complete	—	—	—
0.005 c.c.		0	Distinct trace	Almost complete	—	—	—	—
0.001 c.c.		Trace	Marked	Complete	—	—	—	—

0.025 c.c. Antiserum alone deviated 2 D of Complement.

0.01 " " " " no "

with different amounts of antiserum were practically the same as those fixed with the non-homologous *B. Grünthal* strain. Thus by varying the amount of antiserum used in the complement fixation experiments no demarcation could be demonstrated between, for example, *B. Neapolitanus*, bacillus No. 71, *B. vesiculosus* on the one hand and *B. Grünthal* on the other. This was found to be true also in the case of antisera to bacillus No. 71, *B. coli communis* (Escherich) and *B. vesiculosus*. The procedure of employing a fixed amount of antiserum and varying the amount of bacillary emulsion also failed to elicit any differentiation between a No. 71 strain (other than that used for immunisation) and a *B. coli communis* strain.

When the *B. Grünthal* antiserum was tested in complement fixation experiments along with representatives of typical and atypical coli strains it was found that the group reaction was distinctly limited to a certain class. If coliform bacilli are subdivided as follows :

I. Gas-producing
in sugar fermentation.

II. Non-gas-producing in case
of sugars fermented.

I. 1. Non-inosite-fermenters.

I. 2. Inosite-fermenters,
whether producing indol
or not, *e.g.* *B. lactis*
aerogenes, bacillus No. 67
(MacConkey).

I. 1a. Indol-formers.

I. 1b. Non - indol - formers,
e.g. bacillus No. 74
(MacConkey).

then Sub-class I. 1a comprises most of the typical coliform bacilli, including *B. Grünthal*, *B. vesiculosus*, *B. coli communis* (Escherich), bacillus No. 71 (MacConkey), bacillus No. 106 (MacConkey), *B. Coscoroba*, *B. Neapolitanus*. It was found that the group reaction with the *B. Grünthal* antiserum was limited to these (Table V); thus, with representatives of Sub-class I. 1a (Strain *I*), over fifteen doses of complement were deviated in the presence of 0.025 c.cm. of the antiserum; while with representatives of the other classes in the presence of the same amount of antiserum not more than five doses were deviated, and with some there was no deviation at all (Strains *J*, *K*, *L*, *M*, *N*).

It is noteworthy that some representatives of

TABLE V—COMPLEMENT FIXATION
ANTISERUM TO STRAIN II (*B. GRÜNTHAL*) 0.025 c.c.

Bacillary Emulsion 0.4 c.c.		Strain I.	Strain J.	Strain K.	Strain L.	Strain M.	Strain N.
		Lactose + Adonite + Inulin + Inosite — Indol +	Lactose + Adonite + Inosite + Indol +	Lactose + Adonite + Inosite + Indol —	Coli anaerogenes Lactose + Indol +	Lactose + Inosite — Indol —	Lactose + Inosite — Indol —
Doses of Complement.	3 D	No lysis	Just complete	Very marked lysis	Almost complete	Marked lysis.	No lysis
	5 D	"	Complete	Complete	Complete	Almost complete	Marked lysis
	10 D	"	"	"	"	Complete	Complete
	15 D	"	"	"	"	"	"
	20 D	"	"	"	"	"	"
Complement fixed by Emulsion alone.		4 D	2 D	3 D	2 D	4 D	5 D

TABLE VI—COMPLEMENT FIXATION

Antiserum to Strain II (B. Grünthal).	Bacillary Emulsion.	Doses of Complement.					Complement fixed by Emulsion alone.		
		2 D.	5 D.	10 D.	15 D.	20 D.	2 D.	5 D.	7 D.
0.025 c.c.	0.4 c.c. Strain O. (Non- homolo- gous B. Grünthal). Lactose + Indol +	0	0	0	0	0	Marked	Com- plete	—
0.01 c.c.		0	0	Trace	Distinct	Almost complete	—	—	—
0.005 c.c.		0	0	Very marked	Com- plete	—	—	—	—
0.001 c.c.		Trace	Distinct	Almost complete	—	—	—	—	—
0.025 c.c.	0.4 c.c. Strain F. Lactose — Adonite — Inosite — Indol +	0 0	0 0	0 0	0 Distinct	0 Very marked	Marked —	Complete —	—
0.01 c.c.		0	Trace	Almost complete	Com- plete	—	—	—	—
0.005 c.c.		0	Trace	Almost complete	Com- plete	—	—	—	—
0.001 c.c.		Trace	Distinct	Almost complete	—	—	—	—	—
0.025 c.c.	0.4 c.c. Strain G. Lactose + after mutation. Adonite — Inosite — Indol +	0	0	0	0	0	—	Marked	Com- plete
0.01 c.c.		0	0	0	Trace	Marked	—	—	—
0.005 c.c.		0	0	Distinct	Marked	Com- plete	—	—	—
0.001 c.c.		0	Distinct	Very marked	Com- plete	—	—	—	—
0.025 c.c.	0.4 c.c. Strain H. Lactose + Adonite + Inosite — Indol +	0	0	0	0	0	Marked	Com- plete	—
0.01 c.c.		0	0	0	0	Trace	—	—	—
0.005 c.c.		0	0	Distinct	Com- plete	—	—	—	—
0.001 c.c.		0	Trace	Com- plete	—	—	—	—	—

Class I. 1a, whose fermentative reactions would have delimited them from the accepted types of *B. coli*, deviated in the presence of 0.025 c.cm. of antiserum over fifteen doses of complement, and even by varying the amount of antiserum one could not differentiate them from a Grünthal strain, other than that used for immunisation (Table VI). Included among these were three strains

which, according to the usual criteria, would not have been included in the coli group—namely, two non-lactose fermenters (*e.g.* Strain *F*), and an organism which only fermented lactose after mutation (Strain *G*); these strains all produced indol from peptone. The fact that such Gram-negative bacilli behave in complement fixation experiments with an antiserum to a strain of typical *B. coli*, exactly similar to other typical *B. coli*, strongly suggests that although it has always been considered the most important criterion of a *B. coli*, *lactose fermentation can hardly be taken as of any more importance than other sugar reactions.*

In the course of an examination by complement fixation of thirty typical and atypical coli strains, comprising many different varieties, three indol-forming strains were found to behave in complement fixation with the Grünthal antiserum, like those of the other classes, but no representatives of the other classes, *i.e.* no non-indol-formers, have been found to behave like those of Sub-class I. 1a.

Correlation between Complement Fixation and Resistance to Brilliant Green

It is interesting to quote in this relation experiments on the resistance of different coli types to brilliant green. The great majority of coliform bacill, (see p. 101) are less resistant than *B. typhosus* to this chemical, and include all the typical coli sub-groups, *e.g.* *B. coli communis*, bacillus No. 71, *B. Schafferi*, *B. Neapolitanus*, *B. Grünthal*, *B. vesi-*

culosus, B. Coscoroba, bacillus No. 106 (Mac-Conkey), while certain types less usually met with, particularly inosite fermenters and certain non-indol-formers, exhibit a higher degree of resistance. It is also interesting to note that of the three indol-formers in the complement fixation experiments quoted above, two were distinctly resistant to brilliant green and so were quite unlike the typical coli sub-groups, to which, as regards fermentative reactions, they belong. While exceptions occur there is a distinct parallelism between the grouping of the coliform bacilli one might adopt by a study of their selective resistance to brilliant green and the differentiation obtained by an antiserum such as that used in the complement deviation experiments.

XII

TETANUS

BY C. H. BROWNING

Paper Incorporated

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THE occurrence of muscular spasm following a wound was recognised by the physicians of antiquity, and Aretæus emphasised the gravity of the prognosis in such cases. The comparative rarity of the disease, however, prevented it from attracting wide attention, and extended observations do not appear to have been made until the opportunity was afforded by the wars of the eighteenth century, with their high number of casualties, which included many cases of tetanus. As will be seen later, even provided that the terrain is suitable, it is only war that affords any considerable number of wounds favourable for the implantation of tetanus ; though the employment of fireworks on a large scale, as in the Fourth of July celebrations in the United States of America, has led to very similar conditions and has been productive of many cases.

To-day, knowledge with regard to the pathogenesis

of tetanus is fairly complete, and consequently great advances in therapy, and especially in prophylactic measures, have been made. These all date from the commencement of the bacteriological epoch in the last quarter of the nineteenth century, and are directly due to the employment of animal experiment in the elucidation of disease. Thus, two Italian observers, Carle and Rattone, undeterred by the previous failures of other workers, succeeded in 1884 in producing tetanic symptoms in rabbits, which they inoculated with an emulsion of tissue from the neighbourhood of an acne pustule in a case of tetanus; further, they transmitted the disease by transference from one animal to another. Nicolaier, in 1885, showed that inoculation of mice and other animals with samples of earth caused tetanic symptoms, and he recognised the probable causal agent in a slender bacillus which he grew in mixed culture with other organisms on blood-serum. In 1889, Kitasato obtained pure cultures of Nicolaier's organism under anaerobic conditions; he grew mixed cultures, and then heated these at 80° C. for one hour, by which means the other organisms were killed; the tetanus bacilli, which form resistant spores, survived the heating, and happening to be the only sporing organisms present, they were then obtained as a pure growth when cultures were subsequently incubated in an atmosphere of hydrogen. It was found that although the tetanus bacillus is comparatively uncharacteristic in the unspored stage (a Gram-staining filamentous

organism of variable length, which possesses flagella and in the living state is motile), a highly distinctive appearance is presented when spores are fully developed. Each bacillus then possesses one large, perfectly terminal spore, practically spherical in outline, so that the descriptive term "drumstick bacillus" is extremely appropriate. It must be remembered, however, that tetanus bacilli cannot be recognised with certainty in pathological material, such as the secretions from wounds, by microscopical examination alone; this point is referred to in detail later (see p. 238).

As regards the manner in which the disease-effects are produced, Knud Faber, in 1889, produced tetanic symptoms by the injection of bacterium-free filtrates of impure fluid cultures of *B. tetani* mixed with other organisms; he showed that, as in the case of diphtheria toxin, the filtrates were deprived of their toxic power by heating at 65° C. Kitasato then completed the proof by demonstrating the presence of toxin in sterile filtrates from pure cultures of *B. tetani*.

Under suitable conditions *B. tetani*, when growing in fluid medium, produces one of the most potent soluble poisons known, for a wide variety of animals; thus, the horse, which is exceedingly susceptible, but probably not more than man, can be killed by a subcutaneous injection of 0·003 c.c. of concentrated toxin. In 1890, Behring and Kitasato showed that when an animal was injected with repeated doses of tetanus toxin in non-lethal

amounts, it gradually acquired marked tolerance to the poison; in addition, the blood-serum of such an actively immunised animal neutralised tetanus toxin when mixed with it *in vitro*. The mixture of many times the fatal dose of tetanus toxin with a suitable amount of such "antitoxic" immune serum proved quite harmless when injected into a susceptible animal. This observation constitutes the basis of serum therapy.

In what follows, no attempt is made to deal equally fully with tetanus in all its aspects; but special attention is devoted to those observations, both clinical and experimental, which have a direct bearing on the prophylaxis and treatment of the disease; every effort has been made to avoid omission of any important fact, especially in the light of recent experience, but only representative works have been quoted from the literature.

PATHOGENESIS

SOURCE OF INFECTION.—Tetanus bacilli or their spores are so widely distributed on the earth's surface as to be almost ubiquitous; but they are especially numerous in the dust of roads, in gardens and highly cultivated arable land, and in horse and cattle manure. They have been repeatedly demonstrated in the intestinal contents of horses and cattle, and even of men, especially those working in stables or with horses. Noble found that out of four horses in one stable, which were examined over a period of six months, one showed the presence

of *B. tetani* in cultures made from 0·1 gram of faeces during the first three months, the results thereafter, even with 1 gram of faeces, being negative; a second horse gave positive results with 1 gram in three tests during the first fourteen days, but not again; the other two remained negative throughout. This author therefore concludes that certain horses afford a more favourable habitat for the tetanus bacillus than others, and that in suitable animals multiplication of the organism must occur in the intestine, in order to explain the continued excretion of bacilli; hence these animals are tetanus "carriers." Whether intestinal multiplication is the whole explanation of the prevalence of tetanus bacilli is not clear; but it is certain that the presence of these organisms in horses is variable, since Lukas obtained 16 positive results among 17 horses, while McLeod, Soga, and Roger in this country (unpublished) obtained only 1 positive out of upwards of 30 horses (half of these animals were at a knackery, the others were farm horses working in the neighbourhood of London). Römer found tetanus antitoxin constantly present in the blood-serum of cattle which were over two years old, and as the presence of *B. tetani* in the intestinal contents has been proved by the inoculation of mice with faecal cultures, he considers that adult cattle are, as a rule, the subjects of latent infection with *B. tetani*.

Manure and highly manured soil are therefore pre-eminently the sources of infection with B. tetani

(and also of the other potentially pathogenic anaerobes, the malignant œdema bacilli, *B. Welchii*, etc.) ; but whether the organism flourishes exclusively, or even mainly, inside or outside the animal body is not yet settled. Owing to the resistant character of the spores, infected material may under ordinary conditions continue to be a source of danger for an almost indefinite period except when exposed to direct sunlight.

A wide variety of objects contaminated with tetanus spores have acted as the vehicles of infection, such as splinters of wood, portions of clothing and other foreign bodies, felt used to pad a plaster jacket (Kirmisson), plant fibre—pengawar djambi—employed as a dressing for wounds (Jacobsthal), gelatine, vaccines, diphtheria antitoxin, and possibly catgut and vaccine lymph. Andrewes and Horder have reported tetanus in a woman who had a varicose ulcer of eighteen years' duration, from the surface of which a toxic culture of *B. tetani* was obtained. Contaminated material employed to dress the stump of the umbilical cord appears to explain the heavy incidence of tetanus neonatorum in certain parts of the world, especially in the tropics. With reference to the production of tetanus by anti-diphtheritic serum, it is to be observed that tetanus toxin may be present in the blood of horses some days before they manifest symptoms ; hence harmful effects may follow the administration of a serum which is culturally sterile. Nowadays, precautions are taken to determine that antisera are

free from tetanus spores or toxin by injecting a large amount—10 c.c.—into several guinea-pigs, and also by cultural tests; but, of course, it is possible that the serum may become infected in the course of bottling, after it has been tested.

It is of practical importance to know what procedures will sterilise articles infected with tetanus spores. Thus, Kitasato found steam at atmospheric pressure (*i.e.* 97·5°–98° C.) killed these spores in five minutes (this contrasts with the spores of malignant cedema, which survive heating for an hour under similar conditions); 5 per cent. carbolic acid required fifteen hours to kill; 5 per cent. carbolic acid + 0·5 per cent. hydrochloric acid required two hours; 1 : 1,000 corrosive sublimate required three hours; 1 : 1,000 corrosive sublimate + 0·5 per cent. hydrochloric acid required half an hour.

CONDITIONS OF INFECTION.—The tetanus bacillus and its products are essential for the occurrence of the disease, the characteristic symptoms being due to absorption of the specific soluble toxins which it produces. *B. tetani*, however, is probably quite incapable of obtaining a foothold in the living tissues by its own unaided action. The circumstances which enable it to proliferate in the body under natural conditions are, as a rule, those which promote sepsis—that is to say, a wound contaminated with pyogenic organisms. Further favouring conditions are present where the wound is deep, penetrating—so as not to drain spontaneously—and where foreign matter of any kind

is embedded in the tissues. The presence of much necrotic tissue seems to aid the growth of *B. tetani*; accordingly, lacerated contused wounds are specially liable to be affected; these conditions, of course, also tend to produce sepsis, and so they exert a double action in favouring the onset of tetanus.

Various workers have shown that living tetanus spores, freed from toxin by heating at 80° C., produce no harmful effect when injected subcutaneously into animals; they settle at the site of injection, but some are transported throughout the body, and remain alive for a considerable time in various organs, *e.g.* the liver. Vaillard and Vincent found that such detoxicated tetanus spores, when injected subcutaneously, were actively taken up by phagocytes; hence it is probable that these cells are responsible for the transport of the spores. The spores remain alive, and under favourable conditions are capable of germinating, and by giving rise to toxin may produce fatal tetanus. In guinea-pigs, subsequent injection of living staphylococci into the site at which the spores were introduced, at any time up to one month later, regularly lights up tetanus (Francis). Tarozzi caused tetanus to develop in a rabbit six weeks after the intravenous injection of heated spores, by producing simple fracture of the femur. Semple found viable, virulent tetanus spores at the site of inoculation, six months after subcutaneous injection into guinea-pigs.

Quinine has the same effect as have staphylococci in lighting up a latent tetanus infection, and in this way Semple explained cases of tetanus in men following subcutaneous injections of this drug made presumably with aseptic precautions. He advanced the hypothesis that spores which had by some means entered the tissues at a previous date were enabled to proliferate at the site of the injection of quinine owing to the resulting tissue damage. It is interesting to note that the favouring agencies vary to some extent with the species of animal investigated ; thus staphylococci activate latent tetanus infection in mice, while quinine fails to do so.

The fact that under ordinary conditions of artificial culture *B. tetani* is a strict anaerobe, seems to have obscured somewhat the view of the pathology of the disease, the idea being prevalent that conditions strictly comparable with an anaerobic culture apparatus require to be arranged in the tissues before *B. tetani* will flourish. This has been dispelled by recent work, since even in artificial cultures the organism will grow in bouillon if the minced pieces of flesh are left in the broth, although no growth is likely to occur if a clear filtered specimen of the same broth be employed ; as Tarozzi and Theobald Smith first demonstrated, solid tissue particles promote growth. Again, mixed infection with staphylococci favours the proliferation of *B. tetani* in the tissues, though not *in vitro*, and a number of other organisms exert a

similar favouring action. Whether other anaerobes, like Welch's bacillus and the bacilli of malignant œdema, specially assist *B. tetani* in the tissues is not clear; these organisms are usually present in wounds contaminated with "rich" soil, and, as has been seen, their habitat is the same as that of *B. tetani*. As the present war has shown, shrapnel and bomb wounds afford the combination of conditions specially favourable for tetanus, but a variety of other lesions have led to the disease, such as burns and frost-bite; thus in the present war "trench foot" has led to rapidly fatal general tetanus in many cases, even where there was no obvious breach of surface (Bruce). Although the importance of marked sepsis in the causation of tetanus can scarcely be exaggerated, it must be remembered, on the other hand, that numerous instances have been recorded in which the site of infection was an otherwise trivial wound, which had healed perfectly before the onset of tetanic symptoms; sometimes, but by no means invariably, foreign bodies have been found in the cicatrices. Thus it comes about that *there are no clinical features of a wound pathognomonic of tetanus infection*. Tetanus has also been observed to follow injuries unaccompanied by solution of continuity of the integuments, *e.g.* in cases of simple fracture, hæmatoma, etc. (see Natonek). Accordingly, the presence of aseptic necrotic tissue or trauma, apart from mixed infection, has been considered to afford conditions suitable for the germination of

tetanus bacilli, as in the classical work of Kitasato, who produced tetanus in monkeys by introducing subcutaneously splinters of wood impregnated with tetanus spores; it appears, however, that in most experiments of this kind other organisms also are found at the site of inoculation.

The site of wounds which give rise to tetanus is most commonly on the limbs, first on the legs, then on the arms; the rarity of tetanus complicating wounds of the thorax was noted in the American Civil War. Other paths of entry of the tetanus bacillus apart from wounds have been proved, such as the tonsils and carious teeth, and in one case *B. tetani* was shown by animal inoculation to be present in an abscess of the lung in a young man who died of tetanus when convalescent from pneumonia; also the genital tract affords entrance to the bacillus in women who develop tetanus shortly after parturition. Although, as Ransom showed in guinea-pigs, the healthy alimentary tract has little tendency to absorb tetanus toxin, even when administered in such large amounts that it can be detected in the fæces, it is probable that intestinal lesions, caused by organisms or otherwise, may afford a foothold to *B. tetani*; the complication of typhoid fever with tetanus observed in the wars at the beginning of the nineteenth century is probably to be explained in this way. There remains, however, a certain proportion of "idiopathic" or "rheumatic" cases, said to be most frequent in children and of relatively good

prognosis ("pseudotetanus" of children—Pexa), in which no site of entry of the organism can be determined. In some of these the disease has supervened after exposure to cold or wet, so suggesting that depression of vitality has enabled the bacillus to proliferate and form toxin (in this connection it is noteworthy that Larrey concluded on clinical grounds that exposure to cold and wet favoured the outbreak of tetanus after war wounds); but in others no favouring circumstance could be held responsible for the attack. Whether auto-infection in the sense of absorption of spores from the alimentary canal ever takes place has not been determined; but there appears to be no theoretical ground against this path of entrance of the organism. Spores absorbed in this way might then lie latent in the tissues until infection was lighted up by suitable local conditions.

The question occasionally arises as to whether a wound has been infected with tetanus subsequent to the time of infliction, especially when the incubation period is long. Noetzel's experiments, however, suggest that when once granulation tissue has covered the raw surface, infection with tetanus bacilli is extremely unlikely to occur. Pocketed subcutaneous wounds were made in sheep, and were kept open by packing with gauze without aseptic precautions; when the surface was covered with granulation tissue, in the secretion from which staphylococci were present, a virulent fluid culture of *B. tetani* was poured into the wound, which was

then packed with gauze soaked in the culture. All four animals treated in this way remained alive and well. Thus, granulation tissue, in correspondence with its protective properties in general, seems to afford an almost insuperable barrier to the subsequent entrance of *B. tetani* or its toxins. Conversely, in the course of the present war Nivière has found that operative procedure on a wound was followed in a number of cases by tetanic symptoms in twelve to forty-eight hours, thus suggesting that the onset of the disease had been determined or accelerated by the disturbance of the tissues.

The occurrence of tetanus following surgical procedures—most frequently gynæcological operations—has raised a number of points which have not been satisfactorily settled. E. Koch drew attention to imperfectly sterilised catgut as a source of infection, and he produced tetanus in mice by inoculation with portions of ligature which were found surrounded by pus in a case dying from tetanus after abdominal hysterectomy for impacted fibroids. This work served the useful purpose of emphasising the necessity for securing sterile gut, and the difficulties attendant thereon; it is well known that organisms may survive in gut which has been boiled for half an hour in absolute alcohol. It is probable, however, that a variety of causes are responsible for post-operative tetanus.

It is obvious that the *geographical distribution* of the disease should correspond with the wide distribution of the specific organism. Where the

bacillus is abundant, wounds are, of course, most liable to contamination; on the other hand, where the nature of the soil is such that *B. tetani* is scanty, similar wounds will yield a lower proportion of cases; the relative frequency of tetanus at the beginning of the present war in France and Belgium, and the comparative rarity of the disease in the South African war, are illustrations in point.

As regards *sex incidence*, males are more commonly affected, apart even from war wounds, a fact explicable on the basis of occupation and pursuits. As a rule the disease is sporadic in occurrence, but of course a common source of infection may lead to the outbreak of a series of cases. There is no definite evidence as to whether individuals exhibit marked differences in *susceptibility* to the implantation of tetanus in contaminated wounds. But Dehne and Hamburger, and Aschoff and Robertson, failed to find any notable amount of antitoxin in the blood of normal individuals, thus indicating that natural immunity to the toxin is not common or well marked in the human subject.

TETANUS IN THE HUMAN SUBJECT

In the first place, the disease will be dealt with in the form unmodified by treatment with specific antitoxin. Alterations in the clinical picture due to the administration of serum will be referred to later.

INCUBATION PERIOD.—Although the transport of tetanus spores throughout the body has been proved to occur under certain conditions, this does not invalidate the view that in most cases the symptoms of tetanus are due to toxin produced by bacilli restricted to a particular site, usually a septic lesion. *B. tetani* has been recovered from the lymph glands draining the infected wound, also from the blood, lungs, and spleen in human cases (Reinhardt and Assim); but there is no evidence that a general septicæmic process occurs ordinarily. The symptoms of tetanus appear after a latent stage following infection. According to Rose's collected statistics, symptoms begin in the second week after the infliction of the wound in 46 per cent. of the cases; and in the first analysis of 180 cases treated in home military hospitals (Bruce), over 50 per cent. began in the same period, more cases occurring on the tenth day than on any other; 75 per cent. of the cases manifested symptoms within the first fortnight. While this is the average, remarkable variations in the incubation period are met with, ranging from one or two days to a month or even longer. Where the incubation period is unusually long, the possibility that infection has occurred subsequent to the infliction of the wound must be considered; but as will appear clearly later, a variety of factors determine the duration of the latent stage. For the effect of prophylactic injections of tetanus anti-toxin on the incubation period see p. 260.

SYMPTOMS.—The usual symptoms of a definitely declared attack of tetanus in the human subject are too well known to require more than brief mention; they correspond to the experimental type termed “descending” tetanus (see p. 239). *Increased tonus of the voluntary muscles is the characteristic phenomenon.* This usually commences in the masseters (“lockjaw”), where it is often first observed on waking in the morning after a good night’s sleep; by passing the index finger in between the cheek and the teeth, which are held closed, the tense anterior margin of the masseters can be very well made out. The muscles of the neck are involved practically at the same time. Then the recti abdominis, and later other groups, are affected symmetrically on both sides of the body. Finally, opisthotonus is established, this position being probably due to the relative strengths of the various groups of muscles affected. Pleurosthotonus and emprosthotonus are very exceptional. Associated exaggerated action of the unaffected facial muscles on attempting to antagonise the spastic muscles, as in the effort to protrude the tongue, causes “risus sardonicus.” After the condition has lasted several days, exacerbations of the tonic spasm, *i.e.* tonic convulsions, usually tend to supervene suddenly, without warning, and not as a reflex response; these seizures vary widely in intensity, frequency, and duration (minutes to hours). Again, reflex spasms very frequently follow external stimuli of various kinds,

such as handling the patient, a draught of air, bright light, noise, or in fact any sensory stimulus; or a particular act—for example, swallowing may alone excite the condition (tetanus hydrophobicus). The forearms and hands tend to remain unaffected. The senses and consciousness remain normal as a rule. The spasm of the muscles causes cramp-like sensations of varying severity. Pyrexia may or may not be present, and fever may be accounted for by mixed infection. Profuse sweats are a frequent accompaniment of the exacerbations of spasm. The violence occasionally manifested by the muscular spasms may be appreciated from cases recorded by Anders and Morgan in which the spinous processes of the vertebræ were fractured or the teeth crushed down to the roots. Superficial and deep reflexes are greatly increased, and Goldscheider has frequently observed the Babinski phenomenon (extension of the great toe on stroking the foot sole), either bilateral or confined to the wounded limb.

Sleeplessness is the rule after the tonic spasm has set in, and in cases which progress rapidly to a fatal termination sleep cannot be produced by any means. When sleep occurs—as the result usually of drugs—the spasm relaxes more or less completely, and commonly does not again become declared until several minutes after waking. Green vision has been recorded. Symptoms affecting the other systems are in the nature of accidental complications. Thus, in the period of spasm, retention

of urine and constipation are common; in the convulsions, evacuations tend to occur. Respiratory and cardiac affections are dealt with below (see p. 234). Davies has noticed a tendency to improvement, followed by exacerbation of the symptoms, about the third or fourth day, which may be quite a feature of the disease.

ATYPICAL TETANUS.—Atypical forms of tetanus have been dealt with in great thoroughness by Courtois-Suffit and Giroux. In a small proportion of cases the spasm is at first confined to the muscles of the injured limb, *monoplegic or partial or local tetanus* (see Courtellemont), which may come on within a few days or some weeks after the wound; this corresponds to the sequence of events in animals which have been experimentally inoculated—"ascending tetanus." A degree of this phenomenon is seen when in narcosis the injured limb remains stiff although the other members are relaxed; diagnosis from spasmodic monoplegia of cerebral or medullary origin, incomplete hemiplegia, irritation of motor or mixed nerves, spasms due to lesions of bones, joints, muscles and tendons, tetany and hysterical contractions must be carefully made. On recovery, spasm may disappear last from the injured limb. A distribution of spasm limited to the two upper or lower limbs has also been described—*diplegic or paraplegic form*; when the arm is affected, there is forced flexion of the hand and whole limb; the lower limbs are extended with the feet in the position of equino-

varus. *Cephalic tetanus*: Rose appears to have been the first to emphasise the occurrence of facial paralysis in tetanus following wounds in the distribution area of the facial nerve. The paralysis is usually on the side of the wound, but if the latter is near the middle line it may be bilateral; the paralysis may be total, and there is then hyperacusis and disturbance of taste, or if partial, the superior or inferior branches of the nerve may alone suffer. The mortality in these cases is 50 per cent., *i.e.* below the average; on recovery, paralysis does not persist. In the differential diagnosis of such cases, ordinary nerve injury, hysteria, and tic douloureux must be excluded. Oculomotor paralysis is generally the first symptom of the rare form of tetanus following wounds of the eye or its neighbourhood; diagnosis is difficult, since for several days there may be no other symptoms. The third nerve is almost always involved, but the fourth and sixth are often affected as well (ptosis, strabismus, and more or less complete paralysis of the external muscles); facial paralysis may co-exist with this form. Differentiation from ocular spasms, meningitis (tubercular and meningococcal), fracture of orbit, fracture of base of skull with compression, and traumatic hysteria is necessary. Other forms of cephalic tetanus are the rare non-paralytic type in which trismus and contracture of facial muscles with or without dysphagia occur, also hypoglossal nerve paralysis with the labio-glossopharyngeal paralytic syndrome and associated with

paralysis of the facial nerve, which suggests nuclear origin. See also p. 258.

PREMONITORY SYMPTOMS.—A number of observers have drawn attention to early or “premonitory” symptoms. Where they are recognised, specific serum treatment can be begun without loss of time; the vital importance of avoiding delay in the administration of antitoxin will be discussed later. Where patients are already under observation, the occurrence of premonitory symptoms can, of course, be watched for. A number of observers have recorded the occurrence of pain in the wounded limb, which was taken to be “rheumatic” in character, or of twitching or contracture of the muscles in the affected part, which are felt to be hard when compared with the unaffected side, with or without marked pain. A temperamental change, such as the development of quarrelsomeness, has also been noted. Evler, who himself acquired tetanus, has given an extended list of early symptoms. The following symptoms are significant of early tetanus:

A day or so after the infection there may be general restlessness, changing suddenly to a desire to rest.

Sleeplessness, with distressing dreams and, it may be, nightly delirium.

Difficulty in micturition, due to spasm of the sphincter vesicæ, which may last from a few minutes to half an hour.

Stitch in the side from spasm of thoracic and abdominal muscles.

Temporary giddiness, violent headache, excessive yawning.

The facial appearance changes and the patient looks anxious, though there is no risus sardonius yet,

There may be trembling of the tongue, which is put out to one side.

There is often a profuse sweating, local or general, and darting pains in various parts may occur.

The patient may have a feeling of chilliness, and there may be some swelling, without redness locally, of the injured member, and throbbing of its arteries, notwithstanding that the limb is raised.

Slight jerking may follow pressure on the flexor tendons, and these muscles may be noticed to be in a condition of increased irritability. The deep reflexes may be exaggerated, with knee and ankle clonus. Gibson has pointed out that there is no relation between the condition of the patient as regards his comfort and the state of his reflexes.

If symptoms such as these are present, with a history of possible infection with the tetanus bacillus, Evler considers that the administration of serum is justified. The later symptoms include:

Increased flow of saliva.

Reflex cramps of œsophagus.

Ocular symptoms, *e.g.* nystagmus, strabismus.

Hyperacousis, and spasmodic cough.

Tremors and clonic spasms, which are not painful, and which may not attract attention.

Pain persisting after muscular contraction induced by effort.

With regard to these early symptoms, MacConkey says, "I have carefully read through a considerable number of cases of tetanus described in the literature, and I have found that practically all the symptoms mentioned by Evler have been noticed as being present early in the disease. All have not been observed in the same patient, but usually the one or two present have been sufficiently prominent to force themselves upon the notice of the

physician, and, if he had read Evler's paper, to remind him that such a disease as tetanus might be before him." On the other hand, a criticism which has been passed on these observations is that, on reading them, all would feel that they had those symptoms themselves (Embleton). But when there is the possibility that one may be dealing with so grave a disease as tetanus, it is surely advisable to be on the watch for symptoms such as Evler has described, the significance of which is, of course, unknown to the ordinary patient. The British Official Memorandum on Tetanus emphasises the therapeutic value of detecting such premonitory symptoms; it is specially important that when the wounds are being dressed the adjacent muscles should be palpated in order to detect abnormal hardness (the opposite side or limb serves as the control).

CAUSES OF DEATH.—The most frequent causes of death are asphyxia, exhaustion, and cardiac failure. When an exacerbation of muscular spasm affects the respiratory muscles, especially the diaphragm or glottis, death from asphyxia often occurs; hence cases in which spasm of the glottis or the diaphragm supervenes early are relatively unfavourable; such an attack may, however, cause death at a late date, when diminution in the general spasm indicates convalescence. Œdema of the lungs and pneumonia are liable to set in, and are very dangerous complications. As a result, apparently, of increased peripheral resistance during

the spasm, the heart may come to a standstill either suddenly or gradually. Marked elevation of the pulse-rate is an unfavourable sign. Exhaustion may supervene rapidly; thus, an apparent improvement, in the form of diminution of the trismus, which is often accompanied by otherwise inexplicable hyperpyrexia may be the herald of impending death. The temperature has frequently been observed to continue to rise for about forty minutes post mortem. According to Rose, spasmodic flexion of the arms is a terminal phenomenon. Again, when convalescence appears to be established, patients whose nutrition and general condition have suffered severely may die quite suddenly. No doubt the diminished intake of food and fluid, owing to the trismus, account in part for the exhaustion and wasting, but doubtless the action of the toxin is also directly responsible.

MORTALITY.—All observers agree that tetanus following wounds is an extremely fatal disease; but figures collected from large groups of statistics show marked variations. In general, when the incubation period is less than ten days, the mortality is greater than in cases in which the disease appears later. Permin has investigated with great thoroughness the statistics of the mortality in patients not treated with serum. Six observers who gathered their cases from the literature, found the mortality to range from 17·4 to 50·8 per cent., the figure being under 30 in three series, all Italian. Permin points out that such figures are likely to

be fallacious, on account of the fact that there is a tendency to publish cases which recover or which present unusual features, and to suppress those running the ordinary fatal course. On the other hand, taking the figures of eleven observers in hospital cases, the mortality ranged from 78 to 89·3 per cent. in nine; while the two others, both Italian, gave the mortality as 44 and 34·9 per cent. Permin's own series of 124 cases taken from the records of the larger hospitals in Denmark (and not including tetanus neonatorum), along with Faber's Danish series of 75 cases (each series by itself giving practically the same result), making a total of 199 cases, gave the following figures :

Incubation of 10 days or less,	94 cases ;	deaths = 94·7 per cent.	
„ of over 10 days	57 „	„ = 70·2 „	„
„ unknown	48 „	„ = 58·3 „	„

Thus, the average mortality was 79 per cent. This corresponds well with the general average of hospital figures already quoted. Only the Italians seem to have a much milder type of disease. Rose's collected hospital statistics of cases following wounds, from his own wards, from Great Britain, and in the American Civil War—in all, 716—yielded a mortality of 88 per cent., the extremes being 84·2 and 89·3 per cent.

DURATION OF THE DISEASE.—Four-fifths of the fatal cases die within a week of the onset of symptoms, and over 90 per cent. are dead within fourteen days (Rose). Death after the case has lasted a

month is a rarity. In cases which ultimately recover, the spasm may persist for many months, the masseters being the last muscles to become free. In a case of Rose's, the last exacerbation was observed on the fifty-eighth day of the disease, and trismus was still noticeable on the seventy-fifth day. In another case the exacerbations occurred over a period of five months. Persisting stiffness of the joints of the lower limbs may require treatment by passive movement, as after fracture; it is probable also that impairment of muscular movement results from cicatrices following tearing of the muscle fibres.

PROGNOSIS.—As has been seen under *Causes of Death*, the prognosis in this disease is always extremely doubtful. Those cases in which the spasm sets in after a short incubation period—under ten days—or in which successive groups of muscles become involved in rapid succession (which usually happens when the incubation period is short) are the least favourable. The occurrence of spasm in the diaphragm (pain in the hypogastrium and base of the chest) and glottis (stridor) is in itself a bad omen. But exhaustion, pneumonia, or cardiac failure may supervene unexpectedly at any period. According to Hill's observations, the most important point in prognosis is the time during which the patient has already survived the disease. If the case has lasted less than ten days, the chances of death are greater than of recovery; after ten days of illness, the

chances of recovery progressively increase. Hyperpyrexia is a bad sign, and a temperature over 104° F. on or before the fifth day, practically always means death.

DIAGNOSIS.—*Bacteriological*.—The occurrence of “drumstick,” spored bacilli in the discharge from a wound is suggestive, but by no means conclusive, since other organisms practically identical in appearance with *B. tetani* are met with (M‘Intosh); and *B. tetani* in the non-spored condition is not characteristic. A much more certain procedure than microscopic examination is the production of tetanus in mice, either by the direct subcutaneous injection of secretion from the wound or by filtrates of anaerobic fluid cultures; the latter may give a positive result where direct inoculation fails to produce tetanus (Dean and Mouat), but results are obtained too slowly to be of use for treatment. The cerebrospinal fluid shows no abnormality in the albumin or cell-content, and no coagulum formation (Stintzing; Plaut, Rehn and Schottmüller), points of importance in diagnosis. The demonstration of toxin in the patient’s blood or cerebrospinal fluid when several cubic centimetres are injected subcutaneously into mice, may be successful early in the course of the disease. According to several observers, failure to obtain fluid on entering the subarachnoid space is frequent in cases of tetanus; others again find the pressure of the fluid increased. There is no characteristic leucocytosis.

Clinical Differential Diagnosis.—*Masseter spasm*

occurs in a variety of local lesions—dental abscess, abscess of the temporomaxillary joint, angina, parotitis, irritation associated with the eruption of wisdom teeth—which must be excluded should trismus be the only sign. *Spasm of the neck muscles* occurs in meningitis and cerebral abscess, but trismus is absent in these conditions, and the general features are unlikely to cause confusion with tetanus; in meningitis, too, the cerebrospinal fluid is abnormal. In *tetany*, the marked spasm of the hand muscles, leading to the “obstetric position” of the fingers, is usually a prominent feature; further, the spasm comes and goes, which is not the case in tetanus. *Strychnine poisoning* is characterised by the presence of reflex spasms from the outset, with intervals of relaxation during which, in contrast to tetanus, the masseters and neck muscles also relax completely. *Atypical tetanus* may present considerable difficulty in diagnosis: see pp. 230, 258.

EXPERIMENTAL PATHOLOGY AND SERUM THERAPY

As has been noted, the muscles first affected by spasm in man are usually the masseters, wherever the site of the infected wound may be; thence the affection spreads to other muscular groups—*descending* tetanus. This is the case likewise in the horse and other large animals. In smaller animals, on the other hand, when inoculated with tetanus toxin or bacilli, either subcutaneously or intramus-

cularly, the spasm begins, as a rule, in the muscles of the inoculated limb, and spreads later on to the rest of the body—*ascending* tetanus; with a small dose of toxin, spasm may remain confined to the inoculated limb.

The action of tetanus toxin has formed the subject of a great amount of experimental investigation, the principal contributors being Marie and Morax, Meyer and Ransom, Permin, and Sherrington. The practical as well as the theoretical importance of this work can scarcely be overrated, since upon a clear understanding of the pathology of the disease, as elucidated by the action of toxin and antitoxin under experimental conditions, depends the successful application of antiserum therapy. The following summary of the principal facts and the theoretical considerations to which they have given rise takes no account of the historical development of the subject, as the present comparatively complete state of knowledge depends on the verification and amplification of one observer's work by others.

The local muscular spasm of "ascending" tetanus is due to the action of the toxin on the related segment of the spinal cord. The toxin reaches the cord by passing up the muscular nerves. This has been demonstrated by the following experiments taken in conjunction :

1. Taking advantage of the fact that tetanus toxin when injected into a muscle soon reaches the nerve supplying the muscle, and when in the nerve is practically insusceptible to the neutralising action of antitoxin contained in the blood

and lymph, a rabbit is passively immunised by an intravenous injection of antitoxin, which soon reaches the lymph; then the animal receives a relatively small dose of tetanus toxin into the muscles of the hind leg; the result is that *local tetanus develops in the inoculated limb and nowhere else* (too large a dose of toxin will spread *in the cord*—see p. 245). The same result follows if antitoxin is injected into the thigh muscles and toxin is introduced into the sciatic nerve at the same spot. But if in such a passively immunised animal the sciatic nerve be first cut below the gluteus maximus, and then toxin be injected into the peripheral end, no tetanus occurs. Subcutaneous injection of toxin produces no effect in a passively immunised animal.

The rate of ascent of toxin in the muscular nerve may be judged from the following experiment:—A series of rabbits are immunised intravenously with antitoxin, and then several times the lethal dose of toxin is injected into the leg muscles; at varying times thereafter in different animals, the sciatic nerve on the same side is cut just below the gluteus. It is found that if an interval of five hours or more is allowed to elapse between the injection of the toxin and the neurotomy, local tetanus develops.

If the posterior nerve roots only of one leg are cut, and then later on, after the animal has been passively immunised intravenously, toxin is injected into the muscle of the neurotomed leg, no tetanus follows; a similar injection on the sound side causes spasm as usual. But the injection of toxin into intact posterior nerve roots does not produce tetanic spasm. This is of importance in connection with Sherrington's work (see p. 242).

2. If antitoxin is injected into the sciatic nerve and then toxin is immediately injected into the corresponding limb, tetanus fails to occur in that limb. The intraspinal injection of antitoxin also prevents ascending tetanus.

3. The spasm, when recent, disappears during general narcosis, also on the injection of cocaine intraspinally. On the other hand, after spasm has existed for some time (over thirty hours), changes occur in the affected muscles, for even section of the motor nerve does not then entirely abolish the spasm.

4. Direct injection of toxin into the spinal cord causes tetanus starting, after a very short incubation period, in the muscles innervated from the site of inoculation; but there are also

symptoms of pain, restricted to the part innervated from the site of injection (see p. 246, *tetanus dolorosus*). The extremely short incubation period after direct injection of toxin into the substance of the cord—three to five hours in cats, as compared with twenty-eight to thirty hours after subcutaneous or intravenous injection—leads Meyer and Ransom to attribute the ordinary incubation period in great part to the time taken by the toxin in ascending the peripheral nerves.

The Nature of the Nervous Lesion in Tetanus.—

It has been supposed that the anterior cornual cells of the cord are irritated by the toxin, thus giving rise to increased muscular tonus. These cells are said to exhibit a definite lesion, viz. swelling of the Nissl bodies without disturbance of their arrangement (Flatau and Goldscheider), and recently Manouelian has described other characteristic changes in the motor cells of the cord, pons, and medulla; how far these lesions are the cause of the muscular spasm remains uncertain, however. But it is important to note that there are, in addition, degenerative changes widespread throughout the ganglion cells of the central nervous system, including the cerebral cortex (Foulerton and Campbell Thomson).

According to Sherrington's investigations on animals poisoned by tetanus toxin, there exists a *disturbance of reciprocal innervation*. Thus, stimulation of that area of the cerebral cortex which in a healthy animal causes opening of the mouth—due to contraction of the depressors of the jaw, accompanied by relaxation of the elevators—in a tetanic animal leads to trismus, since the normal

inhibition of the closing muscles, which form the more powerful group, is now converted into a stimulation of them. The condition greatly resembles that of "decerebrate rigidity" in an animal whose cerebral hemispheres have been removed. Clinically, the patient with tetanus exhibits the same phenomena, since the effort to open the mouth, instead of inhibiting the closing muscles, leads to excitation of them, so that his mouth closes all the tighter. The site of the lesion would appear to be in the neighbourhood of the connection between the upper and the lower motor neurons.

The general muscular spasm of "descending" tetanus depends on the diffused action on the nervous system of toxin widely disseminated by the blood and lymph. Even in small animals, when the injection of toxin is made into a non-muscular region, *e.g.* subcutaneously near the knee-joint (Zupnik), general descending tetanus results. Whether the toxin passes to the central nervous system directly from the blood stream, or whether it can reach the nerve centres only by way of the muscular nerves after being taken up from the lymph by the nerve-endings, is a point which has not been satisfactorily determined. After toxin has been injected intravenously, it is possible, by introducing antitoxin into one sciatic nerve, to prevent the occurrence of spasm in this leg; this result may be due not to the antitoxin blocking the ascent of toxin in the nerve from the periphery, but to its having

passed up to the spinal centres and having then exercised its neutralising action in the latter situation.

Those who uphold exclusively the neural route have sought to explain the regular commencement of tetanus in the masseters on the ground of the shortness of the nerve, or of its having a greater affinity for the toxin than other nerves. It is not the case, however, that the sequence of onset of spasm in particular muscles depends entirely on the length of their nerves, since in cats spasm begins in certain muscles of the fore-limbs, the nerves of which are among the longest (Meyer and Ransom). Another view is that the centres of the earliest affected muscles have a special "predilection" for the toxin. A probable explanation of the early appearance of trismus is that the weak action of the antagonising muscles renders the spasm of the masseters obvious (Zupnik); this is in harmony also with the fact that trismus may exist as the only symptom for days before the involvement of other muscles is apparent.

While the muscular nerves act as excellent and rapid conductors, they do not bind the toxin as the central nervous system, especially the grey matter, does (Wassermann and Takaki). The precise mode of conduction in the nerve has not been decided. Marie and Morax, Meyer and Ransom, and others, consider that the axis cylinder is the conductor. In support of their view, Meyer and Ransom quote an experiment in which a rabbit,

very highly immunised to tetanus toxin, died of tetanus as the result of the injection of toxin into the sciatic nerve, its blood—and consequently also the lymph—at the time of death containing more antitoxin in two drops than sufficed to neutralise all the toxin injected. But Field (quoted by Park), and Aschoff and Robertson, hold that the toxin ascends in the neural lymphatics. Experiments showing that toxin injected into a muscle fails to pass into its nerve when the nerve has been cut high up a considerable time previously do not really support the former view; while the fact that toxin which has reached the nerve is insusceptible to neutralisation by the circulating antibodies in an immunised animal can probably be explained by the comparatively slight access of lymph which is possible in that situation; the consequence is that when toxin has once entered the nerve, its action becomes concentrated exclusively on the nervous system. This appears also from the fact that a very much smaller dose of toxin will cause fatal tetanus when introduced intraneurally than when injected into the blood stream or subcutaneously.

When the toxin reaches the cord, it does not remain localised, but spreads; thus, if section of the cord is performed in the upper lumbar region, and afterwards toxin is injected into the sciatic nerve, tetanus remains localised in the hind limbs; whereas if the cord is intact, the anterior part of the body becomes spastic also.

A number of other interesting facts have also been elicited; as, for example, the observation of Meyer and Ransom that an injection of toxin into the posterior nerve-roots between the ganglion and the spinal cord does not produce muscular contraction, but causes severe pain, which recurs in spasms (tetanus dolorosus); the pain is also produced reflexly by slight stimulation of the skin supplied by the affected nerve, as by blowing on it. Again, the injection of an otherwise fatal dose of tetanus toxin into the infraorbital nerve of a dog caused very little effect of any kind—there being in one animal spasm of the ear on the side of the injection, and in another spasm of the masseter. The fact that tetanus dolorosus never occurs clinically, or after intravenous or intraneural injections of toxin, is held by Meyer and Ransom as a proof that, under natural conditions of infection, the toxin reaches the cord only by the axons of the motor nerves, not by lymphatics; the posterior-root ganglion cells are considered to oppose an insuperable barrier to the toxin reaching the cord by sensory nerves. Loewi and Meyer have observed that the injection of a sublethal dose of tetanus toxin into a nerve renders an animal hypersensitive to a subcutaneous dose of the toxin administered some days later; this may have a bearing on the pathology of relapses.

The spontaneous convulsive seizures are probably of cerebral origin. The evidence in favour of this conclusion is as follows:

1. Clinically, in many instances the seizures are not associated with any obvious peripheral stimuli.

2. Roux and Borrel observed that intracerebral injection of tetanus toxin caused epileptiform convulsions; but there remained the possibility that this might be due to the mechanical irritation of the injection. Permin accordingly produced what was in effect an intracerebral injection without this possible fallacy, by introducing the toxin into the vitreous humour in rabbits previously passively immunised by an intravenous injection of antitoxin. The result was the development of recurring universal spontaneous convulsions, with practically no tonic spasm (as has been seen, the latter is produced by toxin acting on the spinal cord).

3. Confirmatory observations made by Permin were: (a) The occurrence of convulsive spasms in the leg of a rabbit which, by means of an intraneural injection of antitoxin, was prevented from sharing the general tonic spasm produced by an intravenous administration of toxin; the non-spastic leg participated equally with the others in the convulsions; (b) In such an animal, the removal of all brain substance from below the corpora quadrigemina upwards abolished the convulsions, whereas, when the animal recovered from the effects of the ether under which the operation had been performed, the general tonic spasm remained marked.

4. As has been noted, marked lesions of the cells

of the cerebral cortex have been found (Foulerton and Campbell Thomson).

Of course, in the naturally occurring disease, both the brain and the spinal cord are exposed to the action of the toxin.

There are a number of further points regarding the behaviour of toxin and antitoxin, and their interaction in the tissues, which have a direct bearing on the prophylaxis and treatment of the disease. Tetanus toxin reaches the blood stream when there is a local infection; this has been proved both in animals and in man, and it has been found that the amount of toxin present in the blood is usually at a maximum before and at the commencement of the tetanic symptoms (McClintock and Hutchings). Toxin also has been demonstrated in the cerebrospinal fluid in human cases (Permin, Stintzing); positive results here are obviously more important than negative. When a fixed amount of tetanus toxin is introduced directly into the blood stream, it disappears rapidly from the circulation, this no doubt being due partly to its passing into the lymph, but also to its combining with certain tissues, especially the central nervous system. Even after the toxin has been removed from the blood, it is, up to a certain point, still capable of being neutralised by a subsequent injection of antitoxin—that is to say, the antitoxin exerts a “curative” effect; but with the lapse of time it becomes more difficult to produce cure. This is proved by the experiments of Dönitz,

who injected a lethal quantity of tetanus toxin intravenously into rabbits; thereafter he injected antitoxin intravenously at increasing intervals, and determined the amount necessary to save life at different times. It was found that when the interval between the injection of toxin and antitoxin was an hour, over ten times as much antitoxin was required as sufficed if the interval was only four minutes.

In applying these results to the treatment of human cases, it is necessary to realise precisely what the conditions are when symptoms have appeared: (1) Toxin has reached the central nervous system, and a certain amount, though probably only a portion, of the toxin has had time to enter into firm combination, and has injured the nerve cells; (2) Toxin is circulating in the blood and lymph as well as in the nerves throughout the body; (3) Toxin is probably still being produced at the site of the wound.

EXPERIMENTAL SERUM THERAPY.—Now, as regards the means at disposal for combating these conditions, toxin in the blood and lymph can be neutralised by antitoxin injected either subcutaneously, intravenously, or intramuscularly. In order that toxin diffused throughout the body may be neutralised, a similar diffusion of the antitoxin must be attained; the subcutaneous injection of antiserum, however, produces this result only slowly, about two days being required before the antitoxin content of the blood reaches a maximum

(as Henderson Smith showed for antidiphtheritic serum, and Dehne and Hamburger for tetanus antitoxin). Hence, in order to ensure rapid neutralisation of the circulating toxin, antitoxin should be administered intravenously ; it has been found experimentally that half an hour after an intravenous injection of antitoxin, both blood and lymph are rendered atoxic (Ransom).

When we come to consider the conditions which are present in the nervous system, there is more difficulty. The central nervous system is not, like the rest of the tissues, bathed by lymph, which in its composition is closely related to blood plasma ; the cerebrospinal fluid which permeates the nervous tissue is peculiar in being extremely poor in protein, and it is well established that antibodies and drugs do not pass readily into it from the blood stream (see Dixon and Halliburton). Hence, however rich the blood may be in antitoxin, whether introduced or as the result of active immunisation, the concentration of antibodies will be low in the fluid surrounding the nerve cells. This explains the fact that a highly immunised animal is susceptible to a comparatively small dose of tetanus toxin introduced directly into the nervous tissue, since it must be remembered that the protective action of antibodies is due to their being present in the surrounding fluid and so neutralising toxin before it can combine with susceptible cells. In order to bring antitoxin into the closest possible contact with the nervous tissue, various workers

have recommended the intraspinal injection of antiserum, and Park and Nicoll have shown experimentally in the clearest fashion the efficacy of this form of treatment. They injected twice the fatal dose of tetanus toxin into the hind leg in guinea-pigs, and seventeen to twenty-four hours later introduced antitoxin by various routes into different animals, viz. subcutaneously, into the general circulation (intracardiac), and intraspinally. The results were as follows :

6 animals were treated subcutaneously—none survived.			
15	„	„	intracardially—2 survived.
16	„	„	intraspinally—13 survived.

Further, the dose of antitoxin which was efficacious when administered intraspinally was much smaller than that injected into the blood stream.

As Andrewes points out, it is really immaterial whether or not these were pure intrathecal injections, since even supposing that a fraction of the antitoxin which was administered entered the spinal cord itself, the fact of the greatly superior efficacy of administration by this route remains. Golla, working with cats and rabbits, has shown that a dose of antitoxin given intravenously or intrathecally is more effective than the same amount administered subcutaneously under similar conditions. Sherrington has lately carried out what is the most important series of observations thus far published on this subject (Bruce, Sherrington); he compared the curative action of

tetanus antitoxin administered to monkeys by the intramuscular and the intrathecal routes. A dried tetanus toxin was employed, of which the fatal dose was less than 0.5 mgm. per kilo of body weight for monkeys. The animals received the dose of toxin, which varied from 1.1 to 4.4 mgm. per kilo as a dry powder into a small cross-cut 8 mm. long by 4 mm. deep cut in the outer head of the left gastrocnemius muscle; the lips of the muscle wound were drawn together by a suture, and the skin wound was closed by stitches—all being done aseptically. The *intramuscular* injections of serum were given into the muscles one-third down the back of the thigh of the same limb as had received the toxin. For the *intrathecal* injections the atlo-occipital membrane was exposed; a needle was passed through it, and after some cerebro-spinal fluid had been withdrawn, serum was then injected without removing the needle. The results are given in the table on p. 253.

The general contrast is especially evident when the fates of animals which received the same dose of toxin per kilo and were treated after practically the same interval, but by the two routes, are compared: *e.g.* Nos. XXXII and XI, VII and XII, XXXV and XXVI, XL and XXXIX; in every instance death occurred after the intramuscular injection, and the course of disease was little if at all protracted, whereas the intrathecal injection of a smaller dose of antitoxin was followed by recovery. Of the group of twelve animals treated

INTRAMUSCULAR AND INTRATHECAL INJECTIONS OF ANTITETANIC
SERUM COMPARED

Monkey's expt. cage number.	Dose of toxin in mgm. per kilo of monkey.	No. of hours from inoculation of—		Dose of serum in units per kilo monkey.	Result.
		Toxin to first symptoms.	Toxin to giving serum.		

<i>Intramuscular</i>					
XXXII .	3.5	29	66	2320	Death in $5\frac{3}{4}$ days.
VII .	2.4	30	66	1360	„ $6\frac{1}{4}$ „
XXXV .	2.0	30	54	1200	„ $10\frac{1}{4}$ „
LXXV .	2.0	30	50	1600	„ $7\frac{1}{2}$ „
LXXVII .	2.0	30	74	2100	„ $5\frac{3}{4}$ „
XXXIII .	1.8	30	69	1480	„ $5\frac{3}{4}$ „
XXIV .	1.7	30	68	2518	„ $11\frac{1}{2}$ „
XLI .	1.6	30	50	2083	„ $5\frac{3}{4}$ „
IV .	1.6	30	72	850	„ $4\frac{1}{8}$ „
XXXVI .	1.5	30	54	1300	„ $12\frac{1}{2}$ „
XL .	1.3	30	74	1365	„ $5\frac{3}{4}$ „
XXXVII .	1.1	30	93	1250	„ $9\frac{1}{2}$ „

<i>Intrathecal</i>					
XVII .	4.4	28	78	1407	Death in $5\frac{1}{2}$ „
XIX .	4.3	28	78	1956	„ $8\frac{1}{4}$ „
XI .	3.5	29	69	1000	Recovery.
XII .	2.7	30	78	910	„
IX .	2.7	30	69	300	Death in $4\frac{7}{8}$ days.
XX .	2.2	30	71	1478	Recovery.
XXIII .	2.1	30	68	1085	„
LI .	2.0	28	51	1500	„
L .	2.0	28	51	1600	„
XXVI .	2.0	30	54	560	„
XLVII .	2.0	30	51	1000	„
LXXVIII .	2.0	30	72	1600	„
LXV .	2.0	30	78	2250	Death in $5\frac{1}{2}$ days.
LVI .	2.0	34	66	1800	Recovery.
XXVIII .	1.9	30	54	711	„
III .	1.8	30	72	966	„
XXXIX .	1.3	30	74	1200	„
XXIX .	1.1	30	93	571	Death in $11\frac{3}{4}$ days

intramuscularly none recovered. Of the eighteen animals treated intrathecally thirteen recovered. As Sir David Bruce says, "Can any unbiased person examine Professor Sherrington's results and not be struck at the manifest superiority of the intrathecal over the intramuscular route?"

In view of these results it is difficult to comprehend how any theoretical consideration should be allowed to stand in the way of the intraspinal treatment of tetanus. It has been objected that antiserum does not enter the nerve cells; but experiments have shown that toxin which has been bound by tissue cells can be removed by contact with a solution containing antitoxin (Besredka, Noon, Graff and Menschikoff). Injection of antiserum into the lateral ventricles has been advised, but does not commend itself on account of the danger of damaging the cerebral tissue; even if this be obviated (Ballance), the method appears unsuited for frequently repeated injections. Again, to inject antitoxin into all the nerves in a case of general tetanus is obviously impossible even if it were desirable, and as Park and Nicoll point out, intraspinal injection probably achieves the same result. The necessity for commencing antitoxin treatment at the earliest possible moment has already been emphasised; the following experiment demonstrates quantitatively the vital importance of attention to this point. MacConkey determined the amount of antitoxin sufficient to save the life of a guinea-pig when injected

under the skin *simultaneously, but at another site*, with a dose of toxin just sufficient to kill an untreated animal; he then found that (1) one-tenth of this amount of serum sufficed to neutralise 100 fatal doses of toxin when toxin and antitoxin were *mixed together in vitro prior to injection*; and (2) two thousand times as much serum was required to save the life of the animal when injected *twenty-four hours after the toxin, i.e.* at a time when symptoms of tetanus had already appeared. In other words, the amount of antitoxin required to cure the animal was two million times as great as the amount which, mixed in the test tube, would render the same dose of toxin inert.¹

PROPHYLACTIC TREATMENT, ESPECIALLY THE USE OF ANTISERUM, AND MODIFICATIONS OF THE DISEASE DUE TO SUCH TREATMENT

Laboratory experiment, as well as veterinary practice, have demonstrated the high value of antitoxic serum—derived from horses immunised by injections of tetanus toxin—for the prevention of tetanus. Thus, Vaillard quotes the result of the preventive inoculation of 13,124 animals during the years 1898–1906 in the practice of eight veterinary surgeons, with no cases of tetanus; while in two other veterinary practices during the same

¹ Tulloch (unpublished) has shown that there are several serologically distinct strains of tetanus bacilli; the bearing of this fact on antitoxin treatment remains to be investigated.

period, 139 cases occurred in untreated animals. Mohler and Eichhorn have determined that 500 U.S.A. units is a sufficient prophylactic dose for a horse, even when infection has occurred four days prior to the injection of antitoxin. In the United States the value of antitoxin in preventing tetanus as a consequence of "fourth of July" injuries has also been clearly apparent; thus, the number of deaths from tetanus fell from 417 in 1903, to 150 in 1909, and 3 in 1914, a reduction "far out of proportion to the reduction in the number of accidents from which tetanus might have resulted" (*Journ. Amer. Med. Assoc.*, No. 26, 1915).

With regard to the prophylactic use of antitoxic serum, the following points require consideration: (1) *Dosage and route of introduction of the serum*; (2) *Duration of immunity thus conferred*.

DOSAGE AND ROUTE OF INTRODUCTION OF ANTITOXIN.—*Dosage*.—There is a consensus of opinion among observers in different countries that for the great majority of wounds 500 to 1000 U.S.A. units¹

¹ Forty U.S.A. units are equivalent to 1 German unit, also known as 1 Behring unit or 1 A.E. The U.S.A. unit of tetanus antitoxin is ten times the least quantity of serum which is required to preserve the life of a 350-gram guinea-pig for ninety-six hours when mixed with the official test-quantity of standard toxin (100 minimum lethal doses) and injected subcutaneously. Commercial sera frequently contain 1500 U.S.A. units in a volume of about 10 c.c. and may consist of "concentrated" antitoxin, *i.e.* a considerable amount of the albumin has been removed by fractional precipitation of the serum. Highly potent or concentrated antitoxic sera containing from 800 to 2000 units or more per c.c. have been prepared for intrathecal administration (see Andrewes).

represent a sufficient prophylactic dose when injected subcutaneously, preferably as soon as possible after the infliction of the wound, or at least within twenty-four hours afterwards. As has been already noted, tetanus infection may occur without obvious solution of continuity of the skin, *e.g.* in "trench foot"; the great importance of administering prophylactic doses in this condition has been emphasised by Bruce. Results obtained during the present war have in this respect confirmed previous experience (see Bruce, also MacConkey's summary of the literature). In the British Official *Memorandum on the Treatment of Wounds in War*, it is stated that there is no evidence that 500 U.S.A. units is an insufficient dose if given promptly. But the broad principle may well be followed, that "as the amount of soiling of the wound and contusion of the tissues increases, so should the amount of antitoxin be increased" (MacConkey).

Route of Introduction of Antitoxin.—It is recommended by some that the injection be made in the vicinity of the wound, so that the toxin may be neutralised before it can diffuse; but in any case, the result of a subcutaneous injection is to render the blood and lymph antitoxic, so that toxin is neutralised before it can reach the nervous system. After a subcutaneous injection the antitoxin content of the blood reaches a maximum in forty-eight hours. This is sufficiently rapid to prevent the outbreak of tetanus in most cases; but occasionally,

whether as the result of the introduction of very large numbers of tetanus spores or of some special favouring condition, symptoms of tetanus may appear in spite of the prophylactic injection. Thus, Wolf records the case of a grenade wound which tore the tendo Achillis from its attachment and splintered the os calcis, and in which the early injection of 20 German units failed to prevent the appearance of trismus thirty hours after the infliction of the wound; the patient received further antitoxin and drug treatment, and recovered. In view of the extremely short incubation period, it is probably safe to say that without the use of antiserum this case would certainly have died. Intravenous administration causes the rapid diffusion of antitoxin throughout the body fluids; accordingly this method would probably prevent a proportion of cases which occur after subcutaneous treatment, and the same dose or even less would suffice.

The local application to wounds of dried antitoxin in the form of a dusting powder has been recommended, and, as the result of experiments, Robertson suggests that a more efficient procedure is to apply a dressing impregnated with antiserum; of course this should not replace the injection of antitoxin.

ATYPICAL TETANUS—THE RESULT OF PROPHYLACTIC ANTITOXIN TREATMENT.—In a number of instances (see Montais, Pozzi) the occurrence of tetanus commencing in the wounded limb, and either spreading to the rest of the body or remaining localised, has been observed in cases where anti-

toxin was administered during the incubation period, but too late to prevent altogether the outbreak of tetanus, *e.g.* two to four days after the infliction of the wound. Thus, the effect is similar to that where an animal is passively immunised and toxin is injected intramuscularly or intraneurally. When the leg is affected in such cases, an early evidence of tetanus is that the foot becomes fixed in the position of equinus, the spasm being aggravated by attempts at voluntary movement or by any local stimulus—the nearer the site of infection that the stimulus is applied the more readily is the spasm evoked (Simmonds, see Burrows); later, the whole limb is rigid, and any stimulus causes painful exacerbation of the spasm (*partial* or *local* tetanus). Such cases of local tetanus in the human subject, often coming on after a long incubation-period (see p. 260), have been much more frequently observed since, during the war, the use of prophylactic injections of tetanus anti-toxin has become general; but the occurrence of local tetanus in untreated cases is not altogether excluded (see Courtois-Suffit and Giroux's monograph on the subject; cases have also been recorded by Burnett and Tulloch, and Ritson). Burrows has pointed out that in such cases when the sole is stimulated the foot and leg are extended, instead of being drawn up, as normally occurs. *Splanchnic tetanus*, of hydrophobic type, without trismus, has been recorded by Burrows.

DURATION OF PASSIVE IMMUNITY CONFERRED

BY A DOSE OF ANTITOXIN.—*The Bearing on Treatment.*—It must be remembered that the passive immunity conferred by an injection of antiserum derived from another species is of relatively short duration, and it has been found, both in the human subject and in animals, that the immunity will have fallen to a comparatively low level after the end of a week following the injection of antitoxin, as shown by the content of the serum in antitoxin (Dehne and Hamburger, Levin). On the other hand, clinical estimates of the duration of protection afforded by a dose of antitoxin based on the date of development of the disease afterwards, yield extremely divergent results (see MacConkey) ; this is readily understood when one remembers how many variable factors are concerned, such as the extent of the wound, the number and possibly the virulence of the tetanus bacilli introduced, the local conditions favouring multiplication of the bacilli and absorption of the toxin, etc. Considering all points, however, a prominent effect of the prophylactic serum treatment is the prolongation of the incubation period which results—up to 365 days (Bruce). As Sir David Bruce says : “ It is the prophylactic injection which governs the incubation period.”

It might be thought that in order to maintain immunity for a long period it would merely be necessary to administer more serum at the beginning. But this is not the case ; the antitoxin disappears largely in virtue of its association with

the foreign protein of the horse serum, which is disposed of by the tissues, and there is no basis for supposing that double the amount will persist in circulation for double the time. Further, the amount of antitoxin which reaches the blood after a subcutaneous injection varies greatly in different persons who have received the same dose, thus indicating that there is also a marked individual factor in the destruction of antitoxin. *Repetition of the subcutaneous dose of antiserum is strongly indicated at weekly intervals so long as the wound is not definitely in a healing stage, and especially if necrotic tissue be still present*, and this has been recommended in the British Memorandum to the extent of a minimum of three injections subsequent to the primary dose at the time of the wound.

Antitoxin does not appear to be effective in leading to the destruction of tetanus bacilli in a wound ; on the contrary, in persons immunised with antitoxin, septic wounds may continue to harbour living virulent tetanus bacilli for many weeks. Antitoxin treatment, therefore, has created many wound carriers of tetanus, who on this account may be a source of danger to others as well as to themselves, and, as in experiments already quoted, so also the human subject may for a long period harbour viable tetanus organisms in his tissues, *e.g.* in a cicatrix ; whether in the absence of prophylactic treatment with antitoxin many individuals may receive *B. tetani* into the tissues at the time of injury and continue to harbour the organism

in a septic wound without developing symptoms of tetanus (as in a case recorded by Dudgeon, Gardner, and Bawtree) is not clear, but there can be little doubt that the employment of antitoxin has greatly multiplied such carriers. The danger which a tetanus carrier himself incurs is due to the fact that his infection does not appear, as a rule, to excite his own tissues to develop any considerable degree of active immunity (Löwy). Hence, if the process of healing of the wound has not led to the elimination of *B. tetani* or the formation of an efficient granulation-tissue barrier against absorption, the effects of the toxin will become manifest when the passive immunity has expired; there is also always the possibility that increased proliferation of the bacilli, accompanied by increased toxin formation, may occur, or suitable conditions for toxin absorption may develop, and so lead to fatal tetanus. These considerations probably afford a full explanation in many cases of the long incubation period of the disease observed in patients who have received prophylactic injections of serum, *e.g.* two months in a case recorded by Don, and five months in a case of Teutschlaender's. In the latter case a grenade splinter passed through the thorax and lodged in the deep muscles of the back. Five months later fatal tetanus developed and led to death within two days. Tetanus bacilli were recovered from the tissue encapsuling the splinter, but not from a chronic empyema due to the perforation of the thorax. Bruce mentions a case

in which the disease broke out after one year. There may also be apparently spontaneous recurrences (Brandt).

The outbreak of the disease at a late stage is specially liable to occur when further operative procedure is undertaken on an infected wound, or even at the site of a healed wound, since the solution of the granulation-tissue barrier permits the spread of infection and the absorption of toxin. Bérard and Lumière have drawn attention to several distinctive features which may be presented by such cases of *delayed tetanus*; thus, the onset may be insidious, the tonic spasm, which may be localised in the wounded limb, progresses very gradually, and trismus and stiffness of the neck may remain slight for days or even weeks; permanent contracture of the abdominal muscles is, however, said to be strikingly constant (late tetanus has been mistaken for intestinal obstruction). Convulsive seizures are absent, or appear late and are slight. In spite of the gradual onset and slow evolution of the disease, however, the prognosis in such cases is grave, death occurring from asphyxia owing to the contracture of the respiratory muscles. *It is, therefore, an excellent rule to administer a prophylactic dose of antiserum before operating on a patient with an infected wound, or before operating at the site of a healed wound; this may be given subcutaneously twenty-four to forty-eight hours beforehand, or if an emergency operation is required, antitoxin should be administered intramuscularly not less than five or six*

hours earlier, or an intravenous injection may be given immediately beforehand. Recrudescence of septic infection also may afford conditions suitable for the occurrence of tetanus in wound carriers. In a case recorded by Huppel, a severe shrapnel wound of the left thigh, with splintering of the bone, was cleaned out five days after infliction, and at the same time a dose of antitetanus serum was given; severe tetanus occurred three weeks later, but recovery took place. Almost four months after the original injury, erysipelas developed from a small area of the wound which alone had remained unhealed; tetanus appeared four days later, and led to death. But even apart from obvious sepsis, trauma at the site of a practically healed wound may lead to an attack of tetanus (Foster).

General Treatment.—The administration of anti-serum after the infliction of a wound does not affect in any way the details of local treatment. It is unnecessary to go into this matter here, beyond saying that whatever method of wound treatment is best in allaying or preventing sepsis will also prove most efficient in obviating conditions suitable for the development of tetanus. It will probably be found that a distinct value attaches to the use of those “disinfectants” which are highly antiseptic and at the same time comparatively harmless to the living tissues, such as the organic chloramides (see Dakin, Cohen, and Kenyon), and also substances like acriflavine and proflavine and brilliant green (see Browning,

Gulbransen, and Thornton, and Leitch). Such benefit as accrues from treatment with peroxide of hydrogen or similar reagents can scarcely be attributed to the inhibitory action which oxygen exerts on anaerobes in artificial cultures.

The use of ultra-violet light has been recommended for the purpose of killing off tetanus bacilli and spores in wounds and cicatrices; but the absorption which these rays suffer in passing through an extremely thin layer of tissue or body fluids is so great (Russ), that little practical result can be expected from the procedure (see p. 154).

INDICATIONS FOR THE USE OF ANTITETANIC SERUM.—All wounds contaminated with “rich” soil, or manure, or into which foreign bodies so contaminated have penetrated, should be regarded as highly suspicious, and the presence of much contused or necrotic tissue should be held to aggravate such suspicion. The presence in a wound of other anaerobic organisms, *B. oedematis maligni* and *B. Welchii*, etc., which have the same habitat as *B. tetani*, also indicates the likelihood that the latter is present. As has been already seen, anti-toxin should be administered at the earliest possible moment, or at least within twenty-four hours. Later on, larger amounts of serum will be required, exactly how much it is difficult to say, but it may be advisable to give up to 10,000 or 15,000 U.S.A. units. The following case (Teutschlaender) is instructive as demonstrating efficacious treatment where the presence of living virulent tetanus bacilli

was proved. The case was one of wound of the foot-sole due to a fragment of grenade. On the sixth day bacilli resembling *B. tetani* were found in the discharge from the wound, and 20 German units of antitoxin were given subcutaneously. Late on the eighth day transient painless contractures occurred in the sole of the wounded foot. Accordingly next day 100 units of antitoxin were injected intravenously; mice inoculated on the eighth day with secretion from the wound developed tetanus. No further symptoms of tetanus occurred; but a guinea-pig inoculated with secretion from the wound on the twenty-fifth day developed tetanus after the long incubation period of fifteen days; two mice inoculated at the same time remained unaffected.

The chances of grave anaphylactic symptoms following repeated subcutaneous injections of serum are fortunately not serious—this matter is treated on p. 273. The further question arises, however, as to whether a case which has already received one or more injections of antiserum is thereby rendered refractory to subsequent passive immunisation; in other words, whether, if a patient is to be treated again with antitoxin, the fact that he has received a previous injection impairs in any way the specific efficiency of the later dose of serum. This point has been investigated for diphtheria antitoxin by Park, Famulener, and Banzhaf in the human subject, under conditions strictly comparable with those likely to arise in practice, and they conclude

that *the same quantity of antitoxin is indicated whether the case has or has not received a previous injection of horse serum or globulins*. There is every reason for believing that their results apply equally to tetanus antitoxin, and this conclusion is borne out by an observation of Dehne and Hamburger on the behaviour of tetanus antitoxin in a man who had been repeatedly injected with horse serum.

· **RESULTS.**—The effect of the extensive prophylactic administration of antitoxin since the earlier days of the present war has had the result of practically abolishing tetanus (see Bruce, also MacConkey). Thus among the wounded sent home to England from overseas the incidence of tetanus was 15·9 and 31·8 per 1000 in September and October 1914, *i.e.* before the introduction of prophylactic inoculation on anything like a complete scale; after the introduction of prophylactic antitoxin treatment the incidence fell abruptly to 1·7 per 1000 in November 1914, and since then has never reached so high a level as 3 per 1000. Both French and German observers are in agreement likewise on this point. It is true also that the organisation for the treatment of wounds has been greatly perfected, and that for this and other reasons the diminution in sepsis might be held to account for the diminished incidence of tetanus; but regarding the extraordinary reduction after “fourth of July” injuries in the United States in the past fifteen years, no corresponding revolution in the treatment of wounds can be discovered.

Again, evidence such as that afforded by Wolf is extremely conclusive, and could no doubt be readily duplicated; this observer deals with the results of a German field hospital which had been stationed several miles behind the trenches at a point on the Western front, where little change of position occurred during the whole period of observation. Prior to December 1914, antitoxin treatment was not a regular procedure, and the cases of tetanus amounted to 1·4 per cent. of the wounded. During the subsequent seven months, for the greater part of which the conditions as regards cold and wet were strictly comparable with the foregoing period, the incidence of tetanus was only 0·16 per cent.; but during this latter time all cases of wounds due to grenades received antitoxin, and also those due to shrapnel where there was evidence of contamination; rifle bullet wounds were not so treated.

THE TREATMENT OF DECLARED TETANUS

It is clear from the pathology of the disease that here one is dealing with an extremely difficult therapeutic problem, since the outbreak of symptoms indicates that toxin has already combined with the central nervous system, and has exerted injurious effects. In cases in which the incubation period is short and in which symptoms develop rapidly, the damage may be irreparable before the patient comes under observation. On the other hand, a

considerable proportion of cases are not of this fulminant type, and in these it is only rational to believe that adequate treatment may turn the scale in favour of recovery. The modes of death and the study of the action of the toxin afford indications for treatment. Uncombined toxin in the body fluids must be neutralised, toxin must, as far as possible, be removed from combination with the nervous tissue by the contact action of concentrated antitoxin, and purely mechanical causes of death, such as asphyxia and starvation due to the muscular spasm, must be obviated. At the same time it is to be remembered that symptomatic remedies which produce muscular relaxation, can exert only a limited beneficial effect; they are themselves for the most part poisons, super-added to that of the bacterial toxin, whose action is not limited to irritating motor neurons. The *tetanus sine tetano* experimentally observed by Dönitz indicates that tetanus toxin produces a general exhausting effect which is ordinarily concealed by the prominent motor symptoms of the acute stage, but which becomes marked when the patient survives for a time.

Where spasm is already present, the subcutaneous injection of serum entails such delay in its action as may render the remedy useless; the rate of absorption is much too slow. Hence, the intravenous route is indicated; by this means free toxin in the tissue fluids will be rapidly neutralised, so that further toxin cannot reach the nervous sys-

tem. This is probably the main value of moderate intravenous doses of antitoxin ; but it is difficult to believe that the intimate proximity of an abundance of antitoxin in the capillary blood-vessels will be altogether without neutralising effect on the toxin in the central nervous system, and this is supported by von Graff's experiments. As has been especially demonstrated by Park and Nicoll, and by Sherrington, however, intraspinal injections of serum are the most effective in antagonising the action of the toxin on the nervous tissue, and a much smaller dose achieves this result by the intraspinal route than by the intravenous. Accordingly, both an intravenous and an intraspinal dose of antitoxin should be given without delay. Where for any reason an intravenous injection cannot be given, the serum should be administered intramuscularly. In cases of *local tetanus* which have been established for some time and in which there is no sign of involvement of the higher centres, it is probably sufficient to give antitoxin intramuscularly or subcutaneously in order to maintain the protection, as these are likely to be patients who have already been treated with antiserum ; but if the case is recent, Andrewes suggests that intrathecal administration may be of benefit, and of course if there is trismus an intrathecal dose should in all cases be given.

With regard to *dosage*, it is certain that much more antitoxin will be required than before the outbreak of symptoms, and even then the result

will be less assured; hence the wastefulness of delay till the disease is declared in cases where there is strong presumptive evidence that infection has occurred. Where the intravenous route alone is employed, a moderate amount of antiserum will neutralise all the toxin which is likely to be in circulation; but probably very large doses are required to influence toxin already present in the nervous tissues. Some observers record the administration of enormous amounts of antitoxin intravenously; thus, Torres has recommended 120 c.c. every six hours. This serves at least to show how much foreign protein can be tolerated, but the expense is almost prohibitive. There is, however, strong experimental evidence against administering a small dose of serum every few hours; the *concentration* attained by the antitoxin is important, hence one large dose of serum should be given. As regards the intraspinal administration of serum, antitoxin rapidly passes out of the cerebrospinal fluid into the blood (Ransom), although the reverse does not hold; hence, to maintain an adequate concentration in the subarachnoid space, repeated injections are required.

SCHEME OF ANTITOXIN TREATMENT—that which is advocated by Park and Nicoll appears to rest on a thoroughly rational basis. It is as follows: Inject, as soon as possible, 3000 to 5000 U.S.A. units intraspinally after lumbar puncture, diluting the serum with sterile normal saline and introducing a volume of 10 to 15 c.c. according to age and

the amount of cerebrospinal fluid withdrawn; the dilution aids thorough diffusion, but where there has been a "dry tap," inject only 3 to 5 c.c. Allow the fluid to run in slowly, by gravity if possible, while the patient is anaesthetised. Give at the same time 10,000 to 15,000 units intravenously. A similar amount injected subcutaneously three or four days later will maintain the tissue fluids rich in antitoxin for the next five days. The intraspinal injections should be repeated at intervals of twenty-four hours for some days (three or four). As regards the intraspinal dose of antitoxin, Andrewes' suggestion that 20,000 to 30,000 units should be given at the outset probably represents an improvement on Park and Nicoll's dosage, since there can be no doubt on experimental grounds that the greater the dose, the higher the chance of success. The preparation of highly concentrated antitoxin (800 to 2000 or more units per c.c.) renders such doses possible. Andrewes and Horder quote a case which gives much support to the intrathecal route—a woman with moderately severe tetanus received 24,000 units of antitoxin intravenously with no benefit; 48 hours later her condition was dangerous and she was given 27,000 units intrathecally. Improvement was manifest next day and she made a good recovery. It has been recommended that for several hours after the intraspinal injection the patient should be kept with the head of the bed lower than the foot, to aid the upward diffusion of the antitoxin; Doyen keeps his patients in this

position, at an angle of 45° for ten hours following each injection. The immediate effect of a dose of serum may be temporary exacerbation of the symptoms, followed later by improvement.

Harmful effects which occasionally follow the administration of serum, especially by the intravenous or intraspinal routes.—These may be grouped under the following headings: (1) *Acquired hypersensitiveness* (anaphylaxis), which is due to a reaction between the tissues and body fluids of the host and foreign protein in the injected serum; this reaction occurs only as the result of repeated injections of serum derived from the same foreign species on each occasion, and is altogether independent of the antitoxin present in the serum; (2) *Primary hypersensitiveness*; and possibly (3) *Effects due to chemical preservatives in the serum*.

1. *Anaphylaxis* is, as a general rule, established ten days after an injection of serum; hence, if a patient has received a previous injection of horse serum, whether as diphtheria or tetanus antitoxin, ten days or more previously, he should not receive a further massive dose of serum intravenously or intraspinally without previous preparation to induce anti-anaphylaxis (see below). Neglect of this precaution has led to serious results, and might cause death.¹ The attempt may be made to detect the

¹ Collapse due to anaphylactic shock following intravenous or intraspinal injections usually appears within a few minutes, and tends to pass off rapidly; the symptoms include convulsive movements, dyspnœa and cyanosis, soft and rapid pulse, low

existence of the anaphylactic state by means of the *allergic reaction*: a small dose of horse serum is injected intradermally (0·1–0·4 c.c.), and if hypersensitiveness is present an urticarial or hyperæmic area one or more cm. in diameter appears in from 5 to 30 minutes (not to be confused with the transient initial swelling due to the introduction of the fluid). A positive reaction indicates the necessity for inducing anti-anaphylaxis; whether a negative reaction excludes all danger of anaphylactic shock is not quite clear. The British Official Memorandum on the use of curative sera points out, however, that if the repeated subcutaneous dose of horse serum is 3 c.c. or less, the danger of anaphylactic shock is negligible; hence this quantity may be repeated at weekly intervals.

2. *Primary hypersensitiveness* leads to symptoms identical with anaphylactic shock; but here the effects follow the first injection. This condition is exceptional, but serious results tend to follow intravenous or intraspinal injections of serum in certain individuals; a history of asthmatic attacks following exposure to emanations from horses should always be inquired for, as this indicates the idiosyncrasy. Thus, if circumstances permit, it is a good rule to take measures to induce anti-anaphylaxis in all cases before administering serum by these routes. The following *method of rapidly*

blood-pressure, pains in head and limbs, feeling of impending death, vomiting, and diarrhoea; coma may supervene, and, in cases terminating fatally, death may not occur for some hours.

inducing anti-anaphylaxis is based on the work of Besredka and of Friedberger : 5 c.c. of the serum are diluted with 50 c.c. of normal saline. Of this mixture 1 c.c. is injected intravenously, and is followed at intervals by further amounts—3 c.c. after four minutes, 10 c.c. two minutes later, 25 c.c. after another two minutes. This fractional introduction of serum can be best effected with the ordinary apparatus for giving intravenous injections by gravitation, the tube connecting the reservoir to the needle being pinched in the intervals. After a further interval of ten to fifteen minutes, the full therapeutic dose of serum may be administered intravenously or intraspinally. An injection of serum given subcutaneously about 24 hours beforehand or intramuscularly a few hours before has also been found experimentally to confer anti-anaphylaxis. There is doubt as to whether these measures are as efficacious in the human subject as in experimental animals (see Dean). But by such means the dangers of hypersusceptibility may be reduced to a minimum and they should not be neglected. Of course, in view of the gravity of tetanus, no consideration of any kind should interfere with the prompt administration of antiserum. Park recommends that the intraspinal injection of serum should be carried out according to Sophian's method, whereby the serum is allowed to run in slowly by gravitation, 30 c.c. requiring over ten minutes to enter. According to Sophian, the blood-pressure should be observed during the

whole operation, and a fall of 20 mm. pressure in an adult indicates that the safe limit of quantity has been reached; British observers, however, do not appear to regard this method of control as a valuable one (*Proc. Roy. Soc. Med., Therapeut. and Pharmacol. Section*, October 19th, 1915). Therapeutic measures which are stated to reduce the dangerous effects of anaphylaxis are ether anæsthesia and injections of atropine sulphate; also adrenalin, of which 0·3 to 0·5 c.c. of 1 : 1000 solution added to 10 c.c. of sterile normal saline may be administered intravenously.

3. *The effect of preservatives in the serum* (0·3 per cent. trikresol being that commonly employed) has been held by some to account for injurious effects following intraspinal injection. Flexner, in discussing the question, held that increased intracerebral pressure and anaphylaxis explained the accidents, since death has followed intraspinal injections of serum free from preservatives (from the Pasteur Institute). The question has been investigated by Worth Hale, Voegtlin, and lately most extensively by Auer; the results obtained vary according to the animal studied and the anæsthetic employed, but the general conclusion clearly follows that trikresol contributes to the toxic action of serum introduced intraspinally. As the respiratory centre is mainly affected, Meltzer's insufflation apparatus should be kept in readiness; and if ill effects follow the introduction of serum, a portion should also be withdrawn at once by gravitation.

The injection of foreign serum intrathecally causes a degree of inflammatory reaction; so that cerebrospinal fluid removed several days after a dose of serum has been given shows the presence of polymorphonuclear cells and increased protein and forms a coagulum. But this reaction does not appear to give rise to any serious effects. For a discussion of "Serum disease" following intrathecal injections, see Rolleston.

Additional treatment comprises thorough cleansing of the wound, adequate special nursing and feeding,¹ avoidance of external stimuli, such as bright light, draughts, noise, unnecessary manipulation of the patient, etc., and the use of drugs which abolish the muscular spasm. The drugs used include practically all the sedatives and narcotics—chloral hydrate by mouth, chloretone in oil per rectum, morphine hypodermically, and ether inhalation (chloroform appears to be less suitable), being especially employed. The dosage, of course, is regulated so as just to secure and to maintain relaxation of the muscles.²

Recently, as a result of Meltzer and Auer's experimental work, magnesium sulphate has been employed to relax the spasm. Magnesium sulphate

¹ Embleton has found constantly aceto-acetic acid in the urine in cases of tetanus, however slight the spasms, and has suggested that it pointed to the possibility of death being due to acidosis; but of course the common cause of acetonuria, viz. relative carbohydrate starvation, must first of all be excluded.

² Average doses are: chloral hydrate 75 to 150 gr., or morphia 1½ gr. daily, chloretone 30 gr. every eight to sixteen hours.

has not come into very general use, even although so eminent an authority as Kocher has warmly advocated the method. The following account of its employment embodies the procedures of Meltzer, and of Kocher. The drug may be administered: (1) Subcutaneously; (2) Intramuscularly; (3) Intraspinally; or (4) Intravenously.

1. *Subcutaneous injection* suffices in milder cases; the dose is 1·2 to 2 c.c. (for a young infant 0·8 c.c.) of a sterile 25 per cent. solution of MgSO_4 crystals (not anhydrous) per kilo of body weight, administered after morphine or under light ether anæsthesia. This represents a quarter of the maximum safe dose, and may be repeated four times in the twenty-four hours for a number of days. The effect is gradual.

2. *By the Intramuscular route* the dose is two-thirds of the subcutaneous, and should be injected into the thigh muscles; the site should then be massaged well. The effect is very potent when ether is used in combination; the patient is etherised before the injection, and a moderate anæsthesia is maintained for twenty minutes afterwards. The action sets in more rapidly, but is more transient, than with subcutaneous injections; on account of the local reaction, repetition is inadvisable.

3. *The Intraspinal method.*—When the case is very severe, with involvement of diaphragm and larynx—a condition most resistant to other treatment—this method produces rapid and prolonged relaxation, which occurs in half an hour and lasts for twenty-four hours. The dose is 1 c.c. of a

25 per cent. solution per 10 kilos of body weight, introduced under ether anæsthesia. The patient should be kept horizontal, with the *head alone raised*, for some hours ; if the thoracic muscles do not relax, the head should be lowered, and it may be necessary to incline the whole body with the feet upward. Deep narcosis results, which may last for twenty-four hours. After the symptoms have been controlled by an intraspinal injection, the attempt should be made to maintain relaxation by subcutaneous administrations.

4. *Intravenous administration* produces the most rapid action ; Straub employs a 2·5 per cent. (*i.e.* isotonic) solution, from 50 to 150 c.c. of which should be injected in two minutes. Spasm relaxes in from one to five minutes, but the effect passes off in half an hour. On account of the effect of intravenous injections on the heart, Meltzer urges caution in the use of this method, and recommends injecting a 6 per cent. solution at the rate of from 2 to 3 c.c. per minute ; slowing or shallowness of respiration is an indication for immediate discontinuance of the injection ; if this does not cause prompt improvement, inject slowly through the venous cannula " a small amount " of 2·5 per cent. calcium chloride.

An objectionable effect which may follow the administration of MgSO_4 is paralysis of respiration, which can be counteracted at once, except in the case of intraspinal administration, by calcium chloride (30 to 40 c.c. of a 2 per cent. solution

intramuscularly, or 500 to 600 c.c. of a 0·2 per cent. solution along with 0·6 per cent. NaCl intravenously); then, of course, the spasm may also recur. An injection of 1·5 mgm. of physostigmine may also be employed, but is probably less safe. Especially in the case of intraspinal administration, arrangements should always be made for insufflation of air or oxygen by Meltzer's apparatus. Kocher states, however, that with the doses mentioned the occurrence of respiratory failure is very rare, and he has not seen a fatality from this cause. Depression of the circulatory function, as shown by softness or intermittency of the pulse, is especially likely to follow intravenous administration, and is a sign for discontinuance; and if the administration is stopped when ill effects commence, they tend soon to pass off. While the necessary precautions have been gone into in detail, it must not be concluded that the method is dangerous; in fact, few remedies are so well under control provided that the administrator knows what preparations are required. Obviously, cases treated in this way must be kept under close observation, and in the use of intraspinal injections the attention must be unremitting. The remarkable property which calcium chloride possesses of immediately awakening an animal narcotised with magnesium sulphate (Meltzer and Auer) suggests that the alteration produced in the nervous tissues is, within limits, a perfectly reversible one; this is a rare and most valuable feature in any drug, and war-

rants a wider trial of the method. The subcutaneous injection of concentrated MgSO_4 is painful at the time, but the pain is said to disappear soon, whereas a weaker solution (5 to 10 per cent.), though painless at first, causes pain later on (Stadler). Zuelzer employs magnesium glycerophosphate, which he states has very little effect on the blood-pressure; safe doses of a 25 per cent. solution are, 10 c.c. intravenously or 5 c.c. intraspinally; intramuscularly, scarcely any pain is produced.

Baccelli's treatment consists in the subcutaneous injection of a 2 or 3 per cent. watery solution of carbolic acid, commencing with the daily dose of 0·3 to 0·5 gram—the urine being observed in order to test the patient's tolerance—and then rapidly increasing to 1 to 1·5 gram phenol daily, administered in repeated injections. A remarkable reduction in the mortality was reported by Baccelli (a collection of individual cases from the literature), which should render this the only method worthy of consideration; but a general confirmation is lacking.

Amputation of the inoculated part has been proved experimentally to serve no useful purpose in the treatment of tetanus (McLintock and Hutchings), and this coincides with the opinion of most clinical observers.

Various heroic measures have been practised as further aids in treatment, such as section of the phrenic nerves.

RESULTS.—From the *statistical aspect*, what

reduction in the mortality from tetanus has resulted from the use of antitoxin? Permin, who has collected what is probably the most satisfactory series of comparable statistics from Danish hospitals—not individual cases compiled from the literature—concludes that antitoxin therapy has an undoubted, although not a pronounced, effect in reducing the mortality. Thus he found that out of 189 cases (not including tetanus neonatorum) treated with serum, 42·3 per cent. recovered, as compared with 21·1 per cent. of recoveries where antitoxin was not used. The details are as follows, and the table of untreated cases is added for comparison :

Incubation.	Cases not treated with Serum.		Cases treated with Serum.	
	Number of Cases.	Percentage of Recoveries.	Number of Cases.	Percentage of Recoveries.
10 days or less	94	5·3	92	27·2
Over 10 days .	57	29·8	57	59·6
Unknown .	48	41·7	40	52·5
Total .	199	21·1	189	42·3

It might be objected that the untreated cases belonged to an earlier period, and that the severity of the disease had altered spontaneously or as the result of other treatment ; but 100 of the untreated cases occurred subsequent to the antitoxin epoch, and of these only 24 per cent. recovered ; they were distributed as follows :

Incubation.	Number of Cases.	Percentage of Recoveries.
10 days or less	47	6·4
Over 10 days	32	37·6
Unknown	21	42·9
Total	100	24

Accordingly, the beneficial effect of antitoxin treatment appears to be clearly established, although this view is by no means universal. A closer analysis of Permin's cases did not establish any advantage following a particular route of introduction of serum, or any other factor. In comment, however, it may be said that, so far, no satisfactory detailed statistics on the antitoxin treatment of tetanus exist, and further, until recently, knowledge as to what is even presumptively the best procedure as to dosage and route of administration has been extremely deficient. It is probably not surprising that the crude mass of British Military Statistics since the outbreak of war (Bruce; Leishman and Smallman; Golla) have not helped to clear up the question of the best method of treatment of declared tetanus. The explanation appears clearly to be the usual one, viz. that in cases of human disease the issue is frequently confused by superadded conditions; thus in the military cases of tetanus there are such highly serious complications as gas gangrene, septicæmia, septic compound fractures, etc. Greenwood on subjecting some of

the records to a statistical analysis has come to the conclusion that the data are altogether inadequate to support any argument in favour of or against the administration of antitoxin by one route or another. On the other hand, the records of series of cases treated by special observers produce a highly favourable impression of the intrathecal method of administration to which, of course, the experimental results of Sherrington and others must be added as a cogent argument for the value of this route. Thus Nicoll's American series of unselected cases, treated by the combined intraspinal and intravenous method of Park and himself, yielded 16 recoveries out of 20 patients ; 9 of the cases had an incubation period of ten days or less, and 6 of these recovered. Doyen, also, has reported 21 recoveries out of 24 cases treated by large intraspinal doses of serum ; 13 of the cases had an incubation period of ten days or less, and 10 of these lived. Andrewes' and Andrewes and Horder's observations have already been quoted, and one of their cases may practically be said to fulfil the conditions of an experiment (see p. 272). Although these series are small, the recoveries seem much too numerous to be the result of chance, and afford the hope that an effective procedure has at length been evolved for the treatment of uncomplicated declared tetanus.

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